THE EFFECTS OF LOCALIZED APPLICATION OF OXYTOCIN AND
VASOPRESSIN IN THE CENTRAL NERVOUS SYSTEM

by

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B.A., University of Arkansas, 1966

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(Department of Physiology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April, 1983
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Date **24 April, 1983**
ABSTRACT

Immunocytochemical studies have demonstrated that nerve fibers containing immunoreactive oxytocin and vasopressin project to many areas of the central nervous system, including the hippocampus and the lateral septum (Buijs, 1980; Sofroniew and Weindl, 1978). Biochemical, physiological and behavioral studies of the effects of these peptides on the CNS have indicated that they are involved in functions as diverse as the control of serotonin turnover (Auerbach and Lipton, 1982), the regulation of body temperature (Kasting et al., 1979) and the retention of conditioned behavior (de Wied et al., 1974; Koob and Bloom, 1982).

The presence of immunoreactive vasopressin (iAVP) in the hippocampus of Wistar rats was confirmed by radioimmunoassay. The vasopressin content of the dorsal hippocampus was $30.3 \pm 7.3$ pg iAVP/mg soluble protein and that of the ventral hippocampus was $81.4 \pm 8.3$ pg iAVP/mg soluble protein, while tissue from the cerebral cortex contained no detectable vasopressin. That this immunoreactivity was due to vasopressin was confirmed by the absence of immunoreactivity in hippocampal or cortical tissue from Brattleboro rats, which are genetically unable to synthesize vasopressin.

Vasopressin applied by iontophoresis was found to increase the activity of neurones in the lateral septum and in the hippocampus of the anesthetized rat. There was no obvious difference between the response of spontaneously active cells and the response of cells excited by continuous iontophoresis of glutamate or acetylcholine. Repeated application of vasopressin resulted in a decline in the magnitude of
the response, but at least part of this decline was due to progressive blockage of the micropipette barrel rather than to tachyphylaxis. Oxytocin, tested only in the septum, was without effect.

When applied by superfusion onto rat hippocampal slices, the NHP peptides were found to increase the activity of 88% of spontaneously active cells and to induce activity in many neurones that were not spontaneously active. Arginine vasopressin, lysine vasopressin, arginine vasotocin, and oxytocin were found to be of roughly equivalent potency, producing a dose dependent response in the range $10^{-9} - 10^{-6}$M. Most cells were tested with more than one peptide and were always found to respond either to all or to none of them. There was no decline in responsiveness when cells were subjected to repeated applications of peptide, but continuous application caused the cells to become unresponsive. Following continuous application of oxytocin, a cell failed to respond to both oxytocin and vasopressin, as would be expected if the two peptides were acting on the same receptor. The analogues ddOT, ddAVP, and Gly\textsuperscript{7}OT were also active, but the oxytocin fragment PLG had no effect, and the vasopressin fragment DGAVP was extremely weak. The response to the peptides could be blocked by vasopressin antagonists.

The peptide sensitive cells appeared to be pyramidal cells rather than interneurones, since the peptide induced activity could be inhibited for about 200-600 msec by electrical stimulation of the stratum radiatum.
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ABBREVIATIONS

Ach  Acetylcholine
ACTH  adrenocorticotropin
AD  receptor mediating the antidiuretic effects of vasopressin
AVP  arginine vasopressin
AVT  arginine vasotocin
B/F  bound/free ratio
BSA  bovine serum albumin
°C  degrees centigrade
cGMP  3',5'-cyclic guanosine monophosphate
CNS  central nervous system
CRF  corticotropin releasing factor
CSF  cerebrospinal fluid
d(CH₂)₅-D-Tyr(Me)AVP  [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-(0-methyl)-D-tyrosine,4-valine]arginine-vasopressin
d(CH₂)₅Tyr(Me)AVP  [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-(0-methyl)tyrosine]arginine-vasopressin
ddAVP  [1-deamino-1,6-dicarba]arginine-vasopressin
dDAVP  [1-deamino-8-D-arginine]vasopressin
ddOT  [1-deamino-1,6-dicarba]oxytocin
DGAVP  [desglycinamide-8-arginine]vasopressin
DGLVP  [desglycinamide-8-lysine]vasopressin
DPM  discharges per minute
dPTyr(Me)AVP  [1-deaminopenicillamine,2-(0-methyl)tyrosine] arginine-vasopressin
EDTA  disodium dihydrogen ethylenediaminetetraacetate dihydrate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>EEG</td>
<td>electroencephalogram</td>
</tr>
<tr>
<td>g</td>
<td>grams or gravities, depending on the context</td>
</tr>
<tr>
<td>Gly&lt;sup&gt;7&lt;/sup&gt;OT</td>
<td>[7-glycine]oxytocin</td>
</tr>
<tr>
<td>HeDI</td>
<td>heterozygous for diabetes insipidus</td>
</tr>
<tr>
<td>HF</td>
<td>receptor mediating the release of hematological factor by vasopressin</td>
</tr>
<tr>
<td>HoDI</td>
<td>homozygous for diabetes insipidus</td>
</tr>
<tr>
<td>iAVP</td>
<td>AVP immunoreactivity</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>I.D.</td>
<td>inner diameter</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>kHz</td>
<td>kiloHertz</td>
</tr>
<tr>
<td>KMNO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>potassium permanganate</td>
</tr>
<tr>
<td>LVP</td>
<td>lysine vasopressin</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MBq</td>
<td>MegaBecquerels</td>
</tr>
<tr>
<td>ME</td>
<td>receptor mediating the milk ejecting action of oxytocin</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<tr>
<td>ml</td>
<td>millilitres</td>
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<tr>
<td>mm</td>
<td>millimetres</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MSH</td>
<td>melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>Ω</td>
<td>megohms</td>
</tr>
<tr>
<td>Symbol</td>
<td>Term</td>
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<td>-------------------------------------------</td>
</tr>
<tr>
<td>nA</td>
<td>nanoamperes</td>
</tr>
<tr>
<td>NHP</td>
<td>neurohypophysial</td>
</tr>
<tr>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>NSB</td>
<td>nonspecific binding</td>
</tr>
<tr>
<td>OT</td>
<td>receptor mediating the uterotonic effect of oxytocin</td>
</tr>
<tr>
<td>OXT</td>
<td>oxytocin</td>
</tr>
<tr>
<td>OVLT</td>
<td>organum vasculosum of the lamina terminalis</td>
</tr>
<tr>
<td>pg</td>
<td>picograms</td>
</tr>
<tr>
<td>PLG</td>
<td>prolyl-leucyl-glycinamide</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>sc</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
</tr>
<tr>
<td>SST</td>
<td>somatostatin</td>
</tr>
<tr>
<td>μA</td>
<td>microamperes</td>
</tr>
<tr>
<td>μg</td>
<td>micrograms</td>
</tr>
<tr>
<td>μl</td>
<td>microlitres</td>
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Finally, I thank Richard Stafford, Christine Nelson, and David Collins, for everything.
CHAPTER I. INTRODUCTION

A. ENDOCRINOLOGY OF THE NEUROHYPOPHYSIAL SYSTEM

1. THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM

The identities of the active principles of the posterior pituitary were established when Du Vigneaud (1956) synthesized the peptides oxytocin and vasopressin and demonstrated that the chemical and biological properties of the synthetic peptides were identical to those of purified pituitary extracts. Ernst Scharrer had proposed as early as 1928 that the hormones were synthesized by neurones in the hypothalamus rather than by the cells in the posterior pituitary itself (Knowles, 1974), but it was not until the 1950's that the concept of neurosecretion became widely accepted. This acceptance was based largely on the work of Ernst and Berta Scharrer and Wolfgang Bargmann, who demonstrated histologically that certain vertebrate and invertebrate neurones contained granular proteinaceous material similar to the material that characterized protein secreting cells elsewhere in the body (Bargmann and Scharrer, 1951). In mammals, this "neurosecretory" material was found to originate in the cell bodies of magnocellular neurones in the supraoptic and paraventricular nuclei and to be transported via the hypothalamo-neurohypophysial tract to nerve terminals in the pituitary (Bargmann and Scharrer, 1951; Bern and Knowles, 1966; Knowles, 1974). Release of oxytocin and vasopressin into the bloodstream involves depolarization of the nerve terminals by action potentials originating in the cell bodies (Cross, 1973; 1974).
2. ENDOCRINE ACTIONS OF THE NEUROHYPOPHYSIAL PEPTIDES

The amino acid sequences of the mammalian neurohypophysial (NHP) peptides are shown in Table I. The posterior pituitary of the adult mammal contains both oxytocin and vasopressin. Oxytocin (OXT), which is released by reflexes originating in the mammary glands and in the reproductive tract, causes milk ejection during suckling and contraction of the uterus during childbirth (Share and Grosvenor, 1974). Vasopressin has both vasopressor and antidiuretic effects and is released by increased plasma osmolarity or decreased blood volume (Schrier et. al., 1979). Vasopressin has recently been shown to increase plasma levels of blood clotting factor VIII and of plasminogen activator (Cash et. al., 1978; Cort et. al., 1981; Mannucci, 1977). This effect is thought to be indirect, with vasopressin promoting release of a hematological factor releasing factor from the brain or pituitary (Cort et. al., 1981).

The vasopressin found in most mammals is arginine vasopressin (AVP), but in the pig and related species and in some marsupials the pituitary contains lysine vasopressin (LVP) instead of or in addition to AVP (Chauvet et. al., 1981). When used experimentally in other mammals, such as the rat and rabbit, LVP has qualitatively the same effects as AVP, although it is less active on a weight basis (Berde and Boissonnas, 1966). Arginine vasotocin (AVT), a neurohypophysial peptide found in non-mammalian vertebrates (Bern and Knowles, 1966; Acher, 1981), is intermediate in structure between oxytocin and AVP. Vasotocin bioactivity has been detected in the pituitaries of fetal mammals (sheep and seals) by Vizsolyi and Perks (1969) and is probably involved in water flow across the amniotic membrane (Vizsolyi and Perks, 1974). Vasotocin may also be present in
Table I. The amino acid sequences of the major mammalian neurohypophysial hormones.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXT</td>
<td>Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH₂</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH₂</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVP</td>
<td>Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-GlyNH₂</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVT</td>
<td>Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-GlyNH₂</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note that the sequences differ only in position 3 (Ile or Phe) and in position 8 (Leu, Arg, or Lys).
adult mammalian brain tissue; Pavel (1971) has reported AVT bioactivity in bovine pineal stalk and subcommissural organ (SCO), and Rosenbloom and Fisher (1975) have found AVT immunoreactivity in rabbit SCO. Vasotocin increases prolactin release and has an antigonadotrophic effect when injected into rats (Blask et al., 1978; Blask and Vaughan, 1980). Recent work by Pevet et al. (1981) however, indicates that the bovine pineal gland contains no AVT, AVP, or OXT; the AVT-like bioactivity was attributed instead to an unidentified peptide which is immunologically similar to AVT and AVP.

The NHP peptides are stored in the pituitary in association with carrier proteins, the neurophysins (Hollenberg and Hope, 1968; Robinson, 1975). It is thought that each nonapeptide and its corresponding neurophysin are fragments of a common precursor molecule (Watson et al., 1982).

Both oxytocin and vasopressin may be involved in the control of the anterior pituitary. Vasopressin has been demonstrated by immunocytochemistry in the median eminence of the rat and monkey (Defendini and Zimmerman, 1978) and is present in high concentrations in the hypophysial portal blood of the monkey (Zimmerman et al., 1973). Vasopressin promotes release of adrenocorticotrophic hormone (ACTH) from the rat pituitary, probably acting in conjunction with another releasing factor (Gilles and Lowry, 1979; Lutz-Bucher et al., 1980). An enzyme in the rat median eminence cleaves oxytocin to release the carboxy terminal tripeptide prolyl-leucyl-glycinamide (PLG, also known as MIF-I), which inhibits the release of melanocyte stimulating hormone (MSH) from the intermediate lobe (Celis et al., 1971; Schwartz and Walter, 1974).
NHP Peptide Receptors

There are at least 5 distinct types of mammalian NHP peptide receptor. As indicated in Table II, the receptors are distinguishable by their different specificities for the various NHP peptides and analogues. The receptors mediating the oxytocic (OT) response in the uterus differ slightly from those mediating the milk ejecting (ME) response in the mammary gland, but both are more sensitive to OXT than to AVP (Berde and Boissonnas, 1966). In contrast, the receptors for vasopressor (VP) activity, for antidiuretic (AD) activity, and for hematological factor (HF) releasing activity are more sensitive to AVP than to OXT (Berde and Boissonnas, 1966; Cash et. al., 1978). The terminology used here for the receptors is based on the corresponding bioassays; the VP receptor has also been referred to in the literature as the smooth muscle (SM) or $V_1$ receptor, the AD receptor as the $V_2$ receptor, and the HF receptor as the HF-R or plasminogen activator (PA) receptor (PA receptor, Cash et. al., 1978; SM, AD, and HF-R receptors, Cort et. al., 1981; and $V_1$ and $V_2$ receptors, Mühlethaler et. al., 1982).

There is evidence that additional types of NHP peptide receptor are present in mammalian tissues. For example, desglycinamide LVP (DGLVP) has been found to produce changes in conditioned behavior and in serotonin turnover (Bohus, 1974; Ramaekers et. al., 1977). Since DGLVP has very little OT, VP, AD, HF, or corticotrophin releasing factor (CRF) activity (de Wied et. al., 1972; Cash et. al., 1978), its effects on behavior and on serotonin turnover indicate the existence of a separate type of vasopressin receptor in the rat CNS. Altura (1975) has demonstrated that there may be at least 3 distinct AVP receptors involved in vasoconstric-
Table II. Relative Potency of NHP Peptides and Analogues on NHP Endocrine Receptors.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>OT</th>
<th>ME</th>
<th>VP</th>
<th>AD</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXT</td>
<td>100</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>AVP</td>
<td>4</td>
<td>14</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>LVP</td>
<td>1</td>
<td>13</td>
<td>68</td>
<td>62</td>
<td>~100</td>
</tr>
<tr>
<td>AVT</td>
<td>26</td>
<td>47</td>
<td>61</td>
<td>62</td>
<td>~50</td>
</tr>
<tr>
<td>dDAVP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>DGAVP/DGLVP</td>
<td>0</td>
<td>?</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Gly7OT</td>
<td>~200</td>
<td>~100</td>
<td>0</td>
<td>0</td>
<td>?</td>
</tr>
<tr>
<td>dDAla9AVP</td>
<td>?</td>
<td>?</td>
<td>0</td>
<td>0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>d(CH2)5Tyr(Me)AVP</td>
<td>ANTI</td>
<td>?</td>
<td>ANTI</td>
<td>0</td>
<td>?</td>
</tr>
<tr>
<td>d(CH2)5DTyr(Me)VAVP</td>
<td>?</td>
<td>?</td>
<td>ANTI</td>
<td>ANTI</td>
<td>?</td>
</tr>
</tbody>
</table>

The activity of AVP at the AD, VP, and HF receptors and of OXT at the OT and ME receptors have been arbitrarily set at 100, and the activities of the other peptides and analogues are expressed as a percentage of these activities. The values in the literature are not entirely in agreement (Sawyer et. al., 1981), and the activities given here should therefore be considered approximations. Inactivity or very weak activity is represented by a "0"; "?" indicates that the information is not available; "ANTI" indicates that the analogue acts as an antagonist. References: Berde and Boissonnas, 1966 (OXT, AVP, LVP, AVT); Cash et. al., 1978 (HF receptor); De Wied et. al., 1972 (DGAVP); Cort et. al., 1981 (dDAla9AVP); Sawyer et. al., 1981 (dDAVP); Lowbridge et. al., 1977 (Gly7OT); and Kruszynski et. al., 1980 and Manning et. al., 1982 (antagonists).
tion; the receptors differ in their relative sensitivities to Phe², Ile³, and Orn⁸ substituted analogues of AVP and are found in different regions of the rat circulatory system (aorta, mesenteric arteriole, mesenteric venule). In addition, the effect of AVT on luteinizing hormone and follicle-stimulating hormone release in the rat (Blask et. al., 1978) and on melanogenesis in the hamster (Logan and Weatherhead, 1981) are mediated by AVT specific receptors. It is also possible that there are specific receptors for the NHP peptide fragments, such as PLG. These additional classes of receptors have not yet been studied in detail and are therefore not included in Table II.

The Brattleboro Rat

The study of the NHP peptides has been greatly facilitated by the discovery that certain rats of the Brattleboro strain are incapable of synthesizing AVP. The condition, characterized by the inability to concentrate urine (diabetes insipidus, DI), is inherited as an autosomal recessive trait; rats homozygous for the disorder (HoDI rats) are completely lacking in AVP, while heterozygous (HeDI) rats have only a partial impairment of AVP synthesis (Valtin et. al., 1965). As would be expected, the chronic polyuria of the HoDI rats results in a variety of electrolyte and hormonal abnormalities, including dehydration, hypokalemia, hypertension, renal damage, increased renin and angiotensin II release, and a reduced pituitary-adrenal response to stress (Bailey and Weiss, 1981). Injection of AVP will block the water loss, but long-term treatment with AVP is needed to restore renal function to normal. The oxytocin content of the posterior pituitary is reduced in HoDI rats, presumably because
Oxytocin is released to compensate for the deficiency of AVP (Valtin et al., 1965). In addition, HoDI rats have an impaired ability to respond to oxytocin (Goren et al., 1980; Hanif et al., 1982). Differences in the β-endorphin content of brain tissues (Pittman et al., 1982), in the regional distribution of opiate receptors (Rigter et al., 1979), and in serotonin and catecholamine metabolism (Kovács et al., 1980) have also been reported.

Brattleboro rats have proved invaluable as a control in immunoassay and immunocytochemical studies, since any immunoreactivity found in HoDI rats is obviously not due to the presence of AVP (Vandesande and Dierickx, 1975; Buijs and Swaab, 1979). The use of HoDI rats as controls in physiological and behavioral studies is less straightforward. It is reasonable to assume, of course, that a response demonstrated in HoDI rats does not depend on AVP - the presence of recurrent inhibition in the HoDI rat, for example, has been cited as evidence that the inhibition is not mediated by AVP (Dreifuss et al., 1973) - but it is more difficult to prove the opposite, that a function found to be impaired in HoDI rats is normally mediated by AVP. This is true both because the defect in the HoDI rat might be an indirect effect of the AVP deficiency, due, perhaps, to hypokalemia or to the disturbed adrenal response, and because one cannot be certain that the lack of AVP is the only genetic defect present in the HoDI rat. The importance of this point in evaluating the role of AVP in behavior (vide infra) has been discussed more fully by Bailey and Weiss (1981), to whom the reader is referred for a more detailed description of the behavioral, metabolic, and endocrine differences between HoDI, HeDI, and normal rats. Comparison of HoDI and normal rats is also compli-
cated by the smaller size and reduced body fat of HoDI rats, and the resulting difficulty in calculating equivalent dosages (Carey and Miller, 1982; Pittman et. al., 1982). In some types of experiments, rats treated with a vasopressin antagonist may be more appropriate controls than HoDI animals.

B. THE NEUROHYPophysial Peptides AND THE CENTRAL NERVOUS SYSTEM

1. EXTRAHYPOTHALAMIC PROJECTIONS OF NEURONES CONTAINING NHP PEPTIDES

The same staining techniques that had been used to demonstrate the hypothalamo-neurohypophysial fibers - the Gomori, aldehyde-fuchsin, and pseudoisocyanine techniques - also revealed hypothalamic fibers projecting to other areas of the central nervous system (CNS), including the septum, the hippocampus, and the habenular region (Bern and Knowles, 1966; Sterba, 1974). These fibers appeared to terminate, not on blood vessels as in the pituitary, but on other neurones. When stained with a KMnO₄ oxidation technique and viewed under the electron microscope, the terminals were found to form synapses not noticeably different from the synapses formed by conventional neurones (Sterba, 1974; Sterba et. al., 1979). These staining techniques, however, were not specific for the NHP peptides (Bern and Knowles, 1966; Sterba, 1974; Buijs, 1980), and the reports did not receive widespread attention.

The development of immunological techniques for staining tissues (Vandesande, 1979) allowed for much greater specificity and sensitivity. Immunocytochemical studies in the rat and monkey revealed that oxytocin and vasopressin were synthesized by separate populations of neurones in
the supraoptic nucleus (SON) and the paraventricular nucleus (PVN), the AVP containing neurones being in the ventral portion of the SON and the medial portion of the PVN (Vandesande and Dierickx, 1975), and by neurones outside these nuclei in areas as distant as the preoptic nucleus, the triangular nucleus of the septum, the substantia innominata, and the zona incerta (Sofroniew and Weindl, 1981; Antunes and Zimmerman, 1978; Kelly and Swanson, 1980). In addition, parvocellular neurones in the supra-chiasmatic nucleus (SCN) were found to contain vasopressin but not oxytocin (Sofroniew and Weindl, 1978; Buijs et al., 1978). The immunocytochemical studies also confirmed the existence of the extrahypothalamic projections. Fibers containing oxytocin project caudally into the medulla and spinal cord, but fibers containing vasopressin are relatively sparse in these areas (Swanson, 1977; Nilaver et al., 1980; Seybold and Elde, 1980). Most of the fibers in the rostral portion of the brain, on the other hand, contain vasopressin rather than oxytocin (Buijs et al., 1978; Buijs, 1980). Terminals immunoreactive for vasopressin have been demonstrated by both light and electron microscopy in the lateral habenula, the lateral septum, the amygdala, and the hippocampus (Sofroniew and Weindl, 1978; Buijs et al., 1978; Buijs and Swaab, 1979; Buijs, 1980). Terminals containing oxytocin have been found in the amygdala and the hippocampus but not in the lateral septum or the lateral habenula (Buijs and Swaab, 1979).

According to Buijs (1980), there are two routes by which fibers immunoreactive for AVP reach the hippocampus: (1) a dorsal projection passing via the fornix and the fimbria to terminate primarily in the ventral hippocampus, and (2) a separate ventral projection passing through the amygdala. The fibers were reported to terminate among the apical and
basal dendrites of the pyramidal cells. Sofroniew and Weindl (1981) have confirmed the existence of the ventral pathway, but the fibers appeared to terminate in the molecular layer of the dentate gyrus and in the hilar region, and they found no evidence of a dorsal projection. The discrepancy between these two studies suggests that the ventral and dorsal projections do not contain the same immunoreactive material. Sternberger (1982) has argued that the immunoreactivity detected in histological studies is not due to the vasopressin and oxytocin molecules themselves, which are lost when the sections are washed, but to larger fragments of the precursor molecules. It is likely that the antisera used by Buijs (1980) and by Sofroniew and Weindl (1981) differ in their affinities for certain of these fragments.

Because of the difficulties involved in tracing individual immunoreactive fibers through several microscope sections, it has not always been possible to determine which hypothalamic nucleus is the origin of a given pathway. Sofroniew and Weindl (1978), for example, attributed the fine fibers in the lateral septum and the thalamus to a projection from the SCN, but a projection from the PVN seems more likely. Lesions of the SCN result in loss of immunoreactivity only in the periventricular nucleus, the dorsomedial nucleus, and the organum vasculosum of the lamina terminalis (OVLT) (Hoorneman and Buijs, 1982), and injection of $^{3}$H-Leucine into the SCN results in very little labelling outside the hypothalamus (Berk and Finkelstein, 1981). A projection from the SON is unlikely, since electrical stimulation of the septum does not cause antidromic excitation of cells in the SON (Poulain et. al., 1980). The caudal projection to the cord and the medulla probably originates in the PVN, since it can
be abolished by bilateral lesions in this nucleus (Nilaver, 1980), and 
since injection of horseradish peroxidase into the cord results in labelling 
of neurones in the PVN (Ono et. al., 1978). According to Buijs (1980), the 
AVP and oxytocin containing fibers in the hippocampus appear to be a pro-
jection of the PVN.

2. EFFECTS OF THE NHP PEPTIDES ON THE CNS

The existence of nerve terminals containing oxytocin and vasopressin 
in the CNS (vide supra) suggests that the peptides can act directly on 
nerve cells. There is considerable evidence that this is the case. The 
biochemical, physiological, and behavioral studies to be described below 
demonstrate clearly that the NHP peptides can affect CNS function, but it 
is not always clear that the peptides were acting directly on receptors 
in CNS tissue rather than on endocrine receptors outside the CNS. Since 
this question is relevant to all the studies to be described below, the 
types of evidence used to distinguish central effects from those secondary 
to changes outside the CNS will be discussed before the studies themselves 
are presented.

That the peptides act directly on CNS tissue is most apparent in 
the in vitro and the iontophoretic experiments, in which actions at 
peripheral receptors would be impossible. The effects of small (1 ng) 
quantities of peptide injected into the cerebral ventricles are also un-
likely to involve peripheral activity, especially if it can be shown 
that much larger subcutaneous (sc) doses are required to produce the same 
responses. That subcutaneous injections can produce the same response 
as intracerebroventricular (icv) injections implies, of course, that
the peptides can cross the blood-brain barrier. While this point is still controversial, recent work in guinea pigs indicates that small quantities of the NHP peptides enter the cerebrospinal fluid (CSF) if the peripheral dose is sufficiently large (Jones and Robinson, 1982). Alternatively, the peptides might be acting on the circumventricular organs or on the anterior pituitary, either of which would be accessible from both the blood and the CSF (Landgraf et. al., 1979; Van Dijk et. al., 1981).

Experiments in which larger quantities of peptide (100 ng or more) are injected into the ventricles should be interpreted with caution, as physiological changes not directly related to neuronal activity may occur. For example, injection of large doses of AVP into the ventricles of monkeys has been reported to cause an increase in the water permeability of brain capillaries (Raichle and Grubb, 1978).

Subcutaneous injections of DGLVP have been found to produce the same changes in conditioned behavior as subcutaneous injections of AVP or LVP (Bohus, 1974; de Wied, 1980). Since DGLVP has little endocrine activity, its effect on CNS function cannot be secondary to vasoconstriction, antidiuresis, or ACTH release. The fact that the DGLVP receptor is distinct from the classical endocrine receptors, however, does not necessarily prove that it is located in the CNS. DGAVP has sometimes been used instead of DGLVP. It is generally assumed that DGAVP is also lacking in endocrine activity, although except for its inability to promote ACTH release (Van Dijk et. al., 1981), this assumption does not seem to have been tested.
Biochemical Studies

Perhaps the best evidence that CNS tissue contains receptors for the NHP peptides is provided by the biochemical changes that can be produced by the peptides in vitro. The oxytocin fragment PLG, for example, has been found to promote cGMP production in a mitochondrial and synaptosomal rat brain preparation in vitro (Spirtes et al., 1980). AVP, at concentrations of $10^{-9}$ to $10^{-6}$M, increased the K$^+$ induced synthesis and release of serotonin from rat hippocampal slices (Auerbach and Lipton, 1982), and both oxytocin and AVP ($5 \times 10^{-7}$ M) reduced the K$^+$ evoked release of $^3$H-dopamine from rat striatal slices (Starr, 1982).

Many of the biochemical changes observed in vivo are also thought to be mediated by receptors in the CNS, although the evidence is usually less direct than with the in vitro studies. Intracerebral microinjections of AVP (50 pg) into certain areas of the rat brain, including the septum and the hippocampus, have been reported to alter catecholamine turnover (Kovacs et al., 1979a; 1979b), and subcutaneous injections of DGLVP (5 μg) can affect the serotonin content of the rat hippocampus (Ramaekers et al., 1977). A deficiency in $^3$H-corticosterone binding capacity has been found in the hippocampus and the anterior pituitary of HoDI rats. This deficiency can be corrected by subcutaneous injections of AVP, dDAVP, or DGAVP (1-2 μg daily for 1 week) but not by oxytocin or ACTH$_{4-10}$ (Veldius and de Kloet, 1982). The mechanism by which vasopressin affects the $^3$H-corticosterone binding capacity and the location of the vasopressin receptor are unknown.
Physiological Studies

Cooper et al. (1979) and Kasting et al. (1979), using push-pull cannulae inserted into the septum of sheep, have reported that immunoreactive AVP is released from the septum during endotoxin-induced fever. Injection of AVP via the cannula reduced the febrile response to endotoxin while injection of anti-AVP serum or of a vasopressin antagonist increased the fever, suggesting that endogenous AVP released by terminals in the septum has an anti-febrile action in sheep. There is also evidence that endogenous AVP may be involved in the response to endotoxin in rats, since systemic injection of endotoxin, which normally causes a fall in body temperature in rodents, produces the opposite reaction in HoDI rats. After chronic pretreatment with subcutaneous AVP, however, the HoDI rats respond normally to endotoxin (Ngsee et al., 1980). Centrally administered AVP (1 µg, icv) not only reduces the body temperature of rats, it can also cause convulsions if given in repeated doses (Kasting et al., 1980). A possible relation between the anti-febrile and the convulsive effects of AVP has been studied by Kasting et al. (1981), who found that both HoDI rats and Long-Evans rats treated with anti-AVP serum (icv) had higher thresholds for heat-induced convulsions than did untreated Long-Evans rats, raising the possibility that AVP released during fever may be partly responsible for febrile convulsions.

Neurophysiological Studies

Peptide induced alterations in neuronal activity have also been reported. In the rabbit, centrally administered LVP (approximately 0.2-20 ng, icv) increased the multiunit discharge rate in the PVN and SON, while
oxytocin had the opposite effect (Schwarzberg et. al., 1974). Injection of DGAVP (20 ng, icv) caused increased electroencephalogram (EEG) theta frequencies in rats, while injection of anti-AVP serum caused a decrease (Urban and De Wied, 1978). Urban (1981) has also reported, however, that as little as $10^{-3}$ pg of AVP or oxytocin reduced the amplitude of theta rhythms when injected directly into the septum of rats. The discrepancy may reflect the difference in dose or in site of injection. Injection of LVP (5-12 ng) directly into the hippocampus of anesthetized rats has been reported to cause hippocampal spreading depression (Huston and Jakobartl, 1977).

A more direct demonstration of the effect of the NHP peptides on neuronal activity has been reported by Nicoll and Barker (1971), who found that iontophoresis of LVP inhibited 80% of antidromically identified supraoptic neurones and excited 90% of cortical neurones tested in the brain of the pentobarbital anesthetized cat. The authors proposed that the AVP released by axon collaterals might be responsible for the recurrent inhibition produced by antidromic stimulation of SON neurones. Recurrent inhibition has also been observed in the rat SON (Dreifuss and Kelly, 1972), but the role of AVP in this phenomenon has been disputed by Dreifuss et. al. (1973), who demonstrated recurrent inhibition in the AVP deficient HoDI rat. While this finding would seem to exclude AVP from consideration as the agent causing the inhibition, the question has not yet been completely resolved. Leng and Wiersma (1981), using more sophisticated techniques, have recently shown that antidromic stimulation caused a reduction of burst length in phasically firing SON cells in Long Evans rats but not in HoDI rats. It would be helpful to determine whether recurrent inhibition in the SON can be blocked by local application of a
vasopressin antagonist.

In contrast to the study by Nicoll and Barker (1971), Moss et. al. (1972) found iontophoresis of AVP to have relatively little effect on antidromically identified neurosecretory cells in urethane anesthetized rats and rabbits; AVP inhibited only one of 6 SON cells tested (species not specified, one SON cell was excited) and inhibited only 2 of 16 rabbit and 13 rat PVN cells (no PVN cells were excited). Unlike AVP, oxytocin excited a majority of antidromically identified PVN cells in both the rat and rabbit brain, but did not excite cells in the SON, cortex, or thalamus. The activity of oxytocin is not confined to neurosecretory cells, however; in a study by Morris et. al. (1980), oxytocin applied by iontophoresis or pressure ejection onto cells in the rat caudal medulla was found to depress neuronal activity in all areas tested (dorsal column nucleus, trigeminal nucleus caudalis, reticular formation, and in the area of the nucleus ambiguus).

Behavioral Studies

Perhaps the most widely used approach to studying the central effects of the NHP peptides has been to determine the behavioral response to central or peripheral injections. Central injections of oxytocin or AVP (100-400 ng, icv) were reported to induce maternal behavior in female rats, AVP being somewhat less effective than oxytocin in this regard (Pedersen et. al., 1982). Central (150-500 ng, icv) or systemic (1500 ng, sc) injections of LVP have been found to have an antinociceptive effect in rats (Kordower et. al., 1981), a finding consistent with the presence of immunoreactive fibers in the substantia gelatinosa of the spinal cord
(Nilaver et. al., 1980). The large doses required in both these studies, however, suggest that the effects may not be centrally mediated. Peripheral injections of the oxytocin fragment PLG potentiate the behavioral effects of L-Dopa (excitement, aggressiveness) in both hypophysectomized and intact mice (Kastin et. al., 1976) and cause more rapid development of morphine tolerance in rats (van Ree and De Wied, 1976). In mice, central injections of the NHP peptides (AVP, LVP, AVT, or oxytocin; 10-180 ng, icv) caused an increase in spontaneous foraging, scratching, and squeaking behavior (Delanoy et. al., 1979). These studies are summarized in Table III.

In some laboratories, however, centrally administered vasopressin produces convulsions or other types of severe motor disturbance. Kruse et. al. (1977) found that AVP, AVT, or LVP injected into the lateral ventricles of rats caused barrel rotation, prostration, and sometimes death. The threshold dose for the production of barrel rotation - a rotation around the longitudinal axis which has been described by Kasting et. al. (1980) as a form of unilateral convulsion - was 8 ng for AVP and AVT and 1.5 ng for LVP. Oxytocin, with a threshold dose of 1 μg, was extremely weak in this test. Similar results were described by Abood et. al. (1980) who found that intraventricular injection of 100 ng of AVP produced seizures in rats. Oxytocin (1 μg) not only failed to produce convulsions but blocked the convulsive effect of a subsequent dose of AVP. Whether oxytocin specifically antagonizes AVP or whether it has a more general anticonvulsant action in this test is not certain, although the finding that oxytocin (∼6 ng, icv) rapidly terminates glutamate induced seizures in rabbits (Schulz et. al., 1974) would support the latter interpretation.
Table III. Behavioral Effects of Neurohypophysial Peptides.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>DOSE</th>
<th>RESPONSE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXT</td>
<td>100-400 ng icv</td>
<td>Maternal Behavior</td>
<td>Pedersen et. al., 1982</td>
</tr>
<tr>
<td>AVP</td>
<td>~8 ng icv</td>
<td>Barrel Rotation</td>
<td>Kruse et. al., 1977</td>
</tr>
<tr>
<td>AVP</td>
<td>~1 μg icv</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXT</td>
<td>10-60 ng icv</td>
<td>Foraging (mice)</td>
<td>Delanoy et. al., 1979</td>
</tr>
<tr>
<td>OXT</td>
<td>180 ng icv</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>100 ng icv</td>
<td>Convulsions</td>
<td>Abood et. al., 1980</td>
</tr>
<tr>
<td>OXT</td>
<td>5 μg icv</td>
<td>Anticonvulsive</td>
<td></td>
</tr>
<tr>
<td>LVP</td>
<td>150-500 ng icv</td>
<td>Antinociception</td>
<td>Kordower et. al., 1981</td>
</tr>
<tr>
<td>LVP</td>
<td>1500 ng sc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>1 μg icv</td>
<td>Immobility</td>
<td>Kasting et. al., 1980</td>
</tr>
<tr>
<td>AVP (3rd dose)</td>
<td>10 ng icv</td>
<td>Convulsions</td>
<td></td>
</tr>
<tr>
<td>AVP</td>
<td>1 ng icv</td>
<td>Memory Retention</td>
<td>Koob et. al., 1981b</td>
</tr>
<tr>
<td>AVP</td>
<td>1 μg sc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The behavioral response to intracerebroventricular (icv) and subcutaneous (sc) injections of the NHP peptides is shown. All experiments were performed with rats except as indicated. In the studies by Kruse et. al. (1977) and by Delanoy et. al. (1979), the dose listed for AVP applies to LVP and AVT as well.
It is not clear why vasopressin produces such extreme responses in some laboratories but not in others. Kruse et. al. (1977), for example, reported a 33% mortality in rats given as little as 40 ng of LVP, while Kordower et. al. (1981) mention only an antinociceptive effect with doses as high as 500 ng. The slight differences in technique between the various studies do not provide an obvious explanation for the discrepancy, and there is no clear evidence that the response varies according to the strain of rat or the commercial source of the hormone.

The response does, however, depend on the previous history of the rat. Experiments involving repeated injections of AVP (Burnard et. al., 1982; Kasting et. al., 1980) have shown that the convulsive action of AVP involves a sensitization process; rats treated with an initial injection of AVP (1 μg, icv) exhibited only minor effects such as immobility, but a second injection 2 days later produced full convulsions, and on the fifth day of the experiment an injection of only 10 ng was sufficient to produce full convulsions. HoDI rats did not differ from normal rats in their initial response but became sensitized more rapidly. The mechanism of this sensitization was not determined. Kasting et. al. (1980) have noted the similarity between AVP sensitization and kindling, a process by which repeated electrical or chemical stimulation of certain brain areas, particularly the amygdala, results in a lowered threshold for convulsions (Girgis, 1981). It is possible that intraventricular AVP stimulates neurones in the amygdala, an area known to be innervated by fibers containing immunoreactive AVP (Buijs and Swaab, 1979), and therefore produces kindling. If this is the case, rats sensitized to AVP should also be more susceptible to seizures produced by other methods of stimulating the
amygdala, a possibility that has not yet been tested. Alternatively, central injections of vasopressin may cause an increase in AVP receptors in the brain, thus rendering the rats more sensitive to AVP but not to other convulsive agents. A general increase in AVP receptors in the brain is unlikely, since the sensitized rats are not more responsive to the hypothermic actions of AVP, but an increase confined to a particular pathway remains a possibility. Whatever the mechanism, one obvious implication of the sensitization is that rats used in behavioral experiments should not be given repeated injections of AVP. Failure to appreciate this fact may account for some of the conflicting dose response relationships mentioned above.

The receptors involved in the production of seizures and related behavior, unlike the classical endocrine receptors described in Table II, appear to be more sensitive to LVP than to AVP. In the study by Abood et al. (1980), for example, LVP was reported to be 3 to 5 times more effective as a convulsive agent than was AVP. Oddly, removal of the glycinamide residue by tryptic digestion - to produce DGLVP and DGAVP - destroyed the convulsant activity of LVP but not that of AVP. It is possible however, that the convulsant activity of the DGAVP was due to contamination with intact AVP. As noted above, Kruse et al. (1977) also found LVP to be more active than AVP in producing barrel rotation, although comparison of the two peptides was difficult because of the "extremely flat" dose response curves. DGLVP was not effective; in fact, Kruse et al. (1977) suggest that DGLVP may have a blocking effect, since LVP did not induce barrel rotation in rats pretreated with a high dose (5 μg, icv) of DGLVP.

There may also be receptors in the brain that respond specifically
to AVT. Pavel has reported that extremely small amounts of AVT ($10^{-18}$ g) injected into the third ventricle of the cat can induce slow wave sleep (Pavel et. al., 1977a) and can reduce plasma cortisol levels (Pavel et. al., 1977b), possibly by acting on neurones in the midline raphé nucleus. Oxytocin and AVP were inactive in this test, even when doses as high as $10^{-6}$ pg were used. In view of the quantities of AVT involved, the reports should probably be viewed with caution.

Effects on Conditioned Behavior

Behavioral studies have also implicated the NHP peptides in the control of memory processes. Posterior lobectomized rats are less resistant than normal rats to extinction of a conditioned avoidance response, but this deficit can be corrected by injection of vasopressin ($\sim 1 \mu g$, sc) (Burbach and de Wied, 1981). Vasopressin is also effective in preventing the amnesia caused by puromycin, $CO_2$, or electroconvulsive shock (Flexner et. al., 1978; Kovács et. al., 1979a). The effect is not confined to rats with a memory defect; vasopressin given to normal rats will also delay extinction of active or passive conditioned avoidance responses (Bohus et. al., 1972; 1978). While the timing of the trials and the injections varies in the different experiments, it appears that the vasopressin is effective when injected shortly before or after (within about one hour) the learning trial or shortly before the retention trial but not if injected at intermediate times. Since vasopressin injected at the time of the learning trial can increase retention measured days later, when the peptide is no longer present, the increase has been attributed to improved consolidation of memory (de Wied, 1980; Krejci et. al., 1979). Rigter (1982) has
argued that the improved consolidation can not be the result of changes in attention, arousal, or motivation, since vasopressin injected immediately after the learning trial is completed has the same effect as earlier injections. The effectiveness of injections given just prior to the retention trial suggests that the peptide also promotes retrieval of memory (Bohus et. al., 1972; 1978; Kovács et. al., 1979a). In most laboratories the action of oxytocin is opposite to that of vasopressin, inhibiting both consolidation and retrieval of memory (Bohus et. al., 1978), but in some experiments oxytocin has been found to mimic the memory promoting actions of vasopressin (van Wimersma Greidanus et. al., 1981).

Intraventricular injections of anti-AVP serum interfere with memory processes, while anti-oxytocin serum enhances memory, as would be expected if endogenous peptides are involved in memory processes (Bohus et. al., 1978; Burbach and de Wied, 1981). The memory defect observed in HoDI rats also suggests a role for endogenous AVP (de Wied, 1980). The vasopressin antagonist dPTyr(Me)AVP (25 μg, sc) antagonizes the effect of exogenous AVP on behavior, and, in higher doses (100 μg) hastens extinction of conditioned responses in rats not treated with AVP (Le Moal et. al., 1981; Koob et. al., 1981a; Koob et. al., 1981b). As would be expected, the oxytocin antagonist Nα-acetyl-[2-O-methyltyrosine] oxytocin enhances conditioned avoidance behavior (Krejci et. al., 1981).

The site of action of the peptides has not been conclusively determined, but the effect on memory is probably not the result of hormonal actions at peripheral sites, since vasopressin injected into the lateral ventricles is effective at much lower doses (1 ng) than vasopressin injected systemically (1 μg) (Koob et. al., 1981b), and since DGLVP produces
the same effects as vasopressin (de Wied et. al., 1972). Microinjections of AVP (50 pg) into the hippocampal dentate gyrus, the dorsal septum, or the dorsal raphé nucleus enhanced performance in a passive avoidance memory test. Oxytocin had the opposite effect when injected into the dentate gyrus or the raphé nucleus but mimicked the effect of AVP in the septum (Kovács et. al., 1979a; 1979b). This work suggests that the response to oxytocin varies according to the site of action and may explain why oxytocin is more variable in its actions than is vasopressin (van Wimersma Greidanus et. al., 1981). Another explanation for the variability observed with oxytocin is that the nonapeptides may be enzymatically degraded to active fragments. According to this view, proposed by Burbach and de Wied (1981), the N-terminal hexapeptide of vasopressin promotes consolidation of memory while the C-terminal tripeptide promotes retrieval. The intact vasopressin molecule, as mentioned above, facilitates both consolidation and retrieval. Both the C-terminal and N-terminal fragments of oxytocin facilitate memory processes (Burbach and de Wied, 1981; Flexner et. al., 1978) and thus have an action opposite to that of the intact oxytocin molecule. The response to an injection of oxytocin would therefore depend on the extent to which the molecule is cleaved in vivo.

This situation is similar to that described by Celis et. al. (1971), in which the effect of oxytocin on MSH release is thought to be controlled by variations in the activity of proteolytic enzymes in the median eminence.

In clinical studies, peripheral administration of vasopressin has been reported to improve memory in humans (Moeglen et. al., 1979; Weingartner et. al., 1981). Changes in mental state (euphoria, well-
being) and in EEG activity have also been reported (Moeglen et al., 1979).

The memory enhancing actions of vasopressin are not an entirely consistent finding. For example, Bohus et al. (1978) reported that vasopressin facilitated memory in a passive avoidance test (latency to enter a box in which a shock had been received 24 hours previously), but Hostetter et al. (1980) were unable to demonstrate any effect of vasopressin in the same test, and Rigter (1980) was able to demonstrate enhanced avoidance behavior only in rats that had been allowed to become familiar with the apparatus before the learning test. Sahgal et al. (1982) found that vasopressin produced a bimodal response - both the number of very short latencies and the number of very long latencies were increased by vasopressin. The bimodal response was said to be more consistent with increased arousal than with improved memory. The memory defect attributed to HoDI rats (de Wied, 1980; Burbach and de Wied, 1981) has been questioned by some workers. HoDI rats were reported by Brito et al. (1981) to learn more slowly than controls, possibly due to greater timidity, but to retain avoidance behavior longer, a finding not consistent with a specific defect in retention. Similar results were reported by Miller et al. (1976), who found that HoDI rats had no defect in retention that could not be accounted for by the delay in learning, and by Carey and Miller (1982), who found that HoDI rats had better retention of passive avoidance behavior than did Long Evans rats. These studies do not argue against a role for the NHP peptides in behavior, but they do suggest that it may be premature to ascribe that role to a specific effect on memory processes.
3. The Present Study

The studies described above provide compelling evidence that the NHP peptides are involved in CNS function, but despite the diversity of these studies relatively little is known about the nature of that involvement. In some types of experiments, particularly those in which the behavioral response to central or peripheral injection of peptide are measured, the reported effects of the peptides are not entirely consistent (Hostetter et al., 1980; Sahgal et al., 1982). This inconsistency is not surprising, since the extensive fiber projections revealed by immunocytochemistry suggest that the peptides are active in many areas of the CNS, and there is evidence that they may produce opposing responses in different areas. For example, oxytocin enhances retention of conditioned behavior when injected into the septum but decreases retention when injected into the dentate gyrus or the dorsal raphe nucleus (Kovács et al., 1979b). An understanding of the functions of the NHP peptides in the CNS will require a more detailed knowledge of the actions of these peptides at individual target areas than is currently available. In particular, it would be useful to know what types of NHP peptide receptor are present in each area. Which areas, for example, respond to oxytocin, and which respond to DGLVP? Do some areas respond to fragments of NHP peptides?

Injection of peptide into the target area, however, is not an entirely satisfactory technique, since the peptide is not properly confined to the site of injection. Kovács et al. (1982) have shown that the micro-injection of anti-AVP serum into the dentate gyrus of the dorsal hippocampus resulted in the spread of antiserum, traced by an immunoperoxidase technique, along the surface of the hippocampus as far caudally as the
ventral hippocampus and rostrally to the lateral septum. Iontophoresis provides a much more localized application of a peptide and allows the response of individual neurones to be monitored, but, until recently, iontophoresis of oxytocin and vasopressin had been attempted only in the hypothalamus and the cortex (Nicoll and Barker, 1971; Moss et. al., 1972). Alternatively, the tissue of interest can be isolated and studied in vitro, but, as of the time that the present investigation was begun, this technique had been used only to demonstrate the presynaptic actions of the NHP peptides.

The present study was undertaken to determine the effect of the NHP peptides on the activity of individual neurones in the septum and the hippocampus of the rat. Both of these areas are known to be innervated by immunoreactive fibers (Buijs, 1980). As described more fully in the previous section of this report, the septum is believed to be the site responsible for the hypothermic actions of vasopressin (Kasting et. al., 1979), and both the septum and the hippocampus have been implicated in the behavioral and biochemical responses to the NHP peptides. The peptides were applied both by iontophoresis in the septum and the hippocampus of the intact rat and by superfusion in the in vitro hippocampus, and the response was monitored by extracellular micropipettes. In addition, the AVP content of the dorsal and of the ventral portions of the hippocampus was measured by radioimmunoassay, since there appears to be a disagreement in the immunocytochemical reports as to whether fibers containing immunoreactive AVP are found in both these areas or only in the ventral hippocampus (Buijs et. al., 1978; Buijs, 1980; Sofroniew and Weindl, 1981).
C. EXPERIMENTAL TECHNIQUES

1. RADIOIMMUNOASSAY

The first radioimmunoassay was developed by Yalow and Berson (1959), who discovered that $^{131}$I-insulin could be quantitatively displaced from insulin-binding antibodies by the addition of unlabelled insulin. The technique was found to be generally applicable, not just to other peptide hormones, but to virtually any antigenic substance. The principle on which the assay is based is relatively simple: unlabelled antigen is allowed to compete with labelled antigen ("tracer") for a limited number of antibody binding sites. The amount of tracer that will bind to the antibody is dependent on the amount of unlabelled antigen which is added; the greater the amount of unlabelled antigen, the smaller the proportion of tracer that will be bound. To determine the concentration of antigen in the sample, the proportion of tracer bound in the presence of the sample is compared to the proportion bound in the presence of known ("standard") concentrations of unlabelled antigen.

The antigen may not be the only substance in the sample that can affect the binding, however. Ions contained in or added to the sample and chemicals used to preserve the sample (heparin, bacteriostatic agents, and enzyme inhibitors) can interfere with the antigen-antibody reaction (Yalow and Berson, 1971a, 1971b). The kinetics of the reaction can also be altered by tissue or plasma proteins which are capable of binding the labelled or unlabelled antigen (Thorell and Larson, 1978, p. 169). Destruction of the antibody or of the label by proteolytic enzymes in the sample will reduce the binding, while destruction of unlabelled antigen will have the opposite effect (Robertson, 1977; Yalow and Straus, 1980).
Interference by salts and chemicals can often be minimized by dissolving the standard in a solution similar in composition to the sample; this is usually feasible only for samples in relatively simple, non-proteinaceous solutions such as CSF. Interfering substances can sometimes be removed by simple separation techniques (dialysis, chromatography), but in many cases it is necessary to use more elaborate purification procedures. Acetone/petroleum ether extraction (Robertson, 1973), for example, is useful for removing proteins and fats from small peptides.

Even if interference by unrelated substances is eliminated, the measurement will be accurate only if the antigen used as a standard is immunologically identical to the antigen present in the sample (Yalow and Berson, 1971b). In the case of the peptide hormones, this identity is often difficult to establish. Species differences in hormone structure, heterogeneity of hormone forms within a species, and the existence of hormone fragments and precursors can complicate interpretation of the experiment or result in conflicting reports from different laboratories (Yalow and Berson, 1971a). For example, if the hormone to be measured is present as part of an immunologically less active precursor, the assay will produce deceptively low values. Conversely, erroneously high values can occur if the sample contains peptides immunologically similar to the hormone in question. For this reason, it is essential to determine the extent to which the antiserum will cross react with any related peptides that may be present in the sample. Several techniques have been developed to demonstrate that the immunoreactive material in the sample is identical to the standard. One can show, for example, that the sample and the standard behave identically in several chromatographic systems. Identity is also
implied, but not proved (Yalow and Berson, 1971a), if serial dilution of 
the sample produces a curve parallel to or superimposable on the standard 
curve. Perhaps the most effective method of establishing the identity of 
the material in the sample is to compare the immunoreactivity of the sample 
with that of similar tissue obtained from hormone deficient mutants or 
from animals surgically deprived of the source of the hormone. The choice 
of techniques will depend on the nature of the sample, the type of inter­
ference anticipated, and the materials available.

2. MICROIONTOPHORESIS

Microiontophoresis has become a well accepted method for applying 
drugs and other substances, such as amines, amino acids, and peptides, 
into the immediate vicinity of the individual nerve cells (Kelly et. al., 
1975). In the most widely used variation of the technique, solutions of 
the compounds to be tested are placed in separate barrels of a multibarrel 
micropipette, the central barrel of which is used to record the activity 
of the cell. The compounds are expelled from the micropipette barrel by 
passing a current of appropriate polarity through the barrel. Cationic 
molecules are expelled by a positive voltage applied to the barrel, while 
anionic molecules are expelled by a negative voltage. Diffusion of the 
compound from the micropipette tip between applications is prevented by 
the passage of a retaining current of opposite polarity to the ejecting 
current. Since the retaining current draws the compound away from the 
tip, there is a delay in release of the compound when the ejecting current 
is applied again (Purves, 1979; Clarke et. al., 1973). Following this 
delay, the rate of release is constant and is usually proportional to
the current flow (Bradley and Candy, 1970; Zieglnsberger et al., 1969). Molecules with a high charge/mass ratio will, in general, be expelled more rapidly than more neutral molecules. The net charge on the molecule can be maximized by adjusting the pH of the solution, cationic molecules being relatively more positively charged at low pH. Much of the current will, of course, be carried by the more mobile $H^+$ and $OH^-$ ions rather than by the compound of interest. Since $H^+$ ions can themselves affect neuronal activity (Frederickson et al., 1971; Gruol et al., 1980), it is necessary to show that the effect observed during iontophoresis of the compound cannot be duplicated by iontophoresis of $H^+$ or $OH^-$ ions from a control solution. Alternatively, one can demonstrate that the effect can be blocked by a specific antagonist. Similar considerations apply to $Na^+$, $Cl^-$, acetate, or any other ions present in the solution.

The release of a compound is sometimes prevented by blockage of the electrode tip. Blockage is indicated by a sharp increase in the resistance of the barrel, by oscillations in the resistance, or by electrical noise (Zieglnsberger, et al., 1969, 1974). Zieglnsberger et al. (1974), in a study of the release of labelled amino acids and amines from iontophoretic pipettes, attributed the blockage of the pipettes to attachment of the compounds to dust and glass particles in the tip. The effect was compared to that of a semipermeable membrane, since current could still be passed through the blocked tip, but the current was carried by small ions instead of by the labelled compounds. Similar results were reported by Purves (1979), who found that sudden increases in resistance caused a complete cessation of release of a fluorescent dye even though current was still passed. Peptides are especially likely to cause blockage
of the pipette tip (Palmer et. al., 1980), presumably because of their relatively low charge to weight ratio and their tendency to stick to glass. Naturally, effects observed during the passage of current through a blocked pipette cannot be attributed to the drug or peptide being studied.

3. THE HIPPOCAMPAL SLICE

The transverse hippocampal slice, first developed by Skrede and Westgaard (1971), has quickly become one of the most popular neurophysiological preparations available. One reason for this popularity is the well defined anatomical structure of the hippocampus. As illustrated schematically in Figure 1, this structure is preserved in the hippocampal slice. The two major cell types are arranged in layers, the pyramidal cell layer of the hippocampus proper (divided into regions CA1 to CA4) and the granule cell layer of the dentate gyrus. Since these layers are readily visualized under low power magnification in vitro, the electrodes can be positioned more easily and with greater accuracy than is possible with stereotaxic techniques. Interneurones are also present in the hippocampus; these are located primarily in or near the pyramidal cell layers (Lee et. al., 1980), but scattered cells are found throughout the hippocampus (Shepard, 1974; Gall et. al., 1981). Furthermore, the major fiber projections within the hippocampus - the perforant path projection to the granule cells, the mossy fiber pathway from the granule cells to CA3, and the Schaffer collateral pathway from CA3 to CA1 - are all aligned roughly parallel to the parasagittal plane (Lømo, 1971). If the hippocampus is cut along the same plane (Figure 1B), the projections will remain intact in the slice preparation. In the present experiment the Schaffer collateral
Figure 1. The anatomy of the rat hippocampal slice.

(A) Schematic drawing of the hippocampal slice as it appears under low power magnification. The pyramidal cell layer (triangles) and the granule cell layer (circles) are indicated, and representative cell types have been sketched to indicate the orientation of the dendrites. Only those projections discussed in the text are shown. (B) Schematic drawing of the hippocampus as seen from above, to show the orientation of the slice. Figures adapted from Skrede and Westgaard, 1971; Shepard, 1974; and Dingledine et al., 1980.
pathway was stimulated by an electrode in the stratum radiatum, and the response was monitored with an electrode in the cell body layer of area CA1 (Figure 2). The evoked field response (Figure 2B) includes a broad positive potential, due to the summed post synaptic potentials in the dendrites, and a sharp negative population spike resulting from the summed action potentials in the cell body layer. The response of a single pyramidal cell (Figure 2C) is usually a single action potential followed by a period of inhibition.

The cells in the in vitro preparation have a relatively constant and controlled environment. Since there is, of course, no blood flow, there are no changes in blood pressure or in the concentrations of hormones or anesthetics in the blood. The absence of vascular and respiratory pulsations has made the slice particularly useful for intracellular recording. The extracellular space is directly accessible to experimental manipulation, so that transmitters, hormones, and pharmacological agents can be added without the impediment of a blood brain barrier. Special techniques for applying the substances, such as iontophoresis or pressure ejection, are required only if the application is to be restricted to a particular portion of the slice. The accessibility of the extracellular space also means that the behavior of the cells will be dependent, in part, on the ionic composition of the bathing medium, the excitability of the cells being increased by a high K⁺ or a low Ca²⁺ concentration. This is an advantage in the sense that the experimenter can choose the optimum conditions for a particular type of experiment, but it is impossible to say what conditions produce the most "normal" behavior (Schwartzkroin, 1981). Most workers find that a medium with relatively high concentrations of
Figure 2. Position of electrodes for recording the response to Schaffer collateral stimulation. (A) Schematic drawing of hippocampal slice, as in Figure 1, showing the position of the electrodes for recording the response of CA1 pyramidal neurones to stimulation of the Schaffer collateral pathway. (B) Field potential produced by Schaffer collateral stimulation, recorded with a filter of 0.1-3 kHz. (C) Single unit response to stimulation of the same pathway, recorded with a filter of 1-3 kHz.
both $K^+$ (5-6 mM) and $Ca^{++}$ (2.0 mM) produces a more stable preparation, especially for intracellular recording, than does a medium containing more physiologically "correct" concentrations. This belief has been questioned by Dingledine et. al. (1980), who report that suitable recordings can be made with artificial CSF containing 3.0-3.5 mM $K^+$ and 1.2 mM $Ca^{++}$.

The chief disadvantage of the *in vitro* preparation is that the tissue is inevitably damaged in cutting - the axons of all the pyramidal cells and of some of the granule cells and interneurones will be severed, and many cells will have severed dendrites as well. Intracellular recordings indicate, however, that the membrane properties of the cells (resting membrane potential and conductance) are usually quite similar to those measured *in vivo* (Schwartzkroin, 1977). The loss of the afferent projections to the hippocampus will alter the behavior of the cells in ways that cannot be easily predicted. In addition, the slices are subjected to a period of anoxia and to considerable mechanical trauma during dissection and cutting. A further decline in tissue viability occurs during the course of the experiment, although this deterioration is usually very gradual; slices have been kept alive as long as 40 hours under sterile conditions (Swanson et. al., 1982). The condition of the slice can be judged from the electrophysiological responses, particularly by the loss of inhibition with double pulse stimulation and the appearance of multiple population spikes, and by the visual appearance of the slice (Dingledine et. al., 1980; Teyler, 1980).

While the *in vitro* hippocampal slice is an extremely useful preparation, permitting experimental manipulations that would not be feasible
in vivo, it should be remembered that even the healthiest slice is grossly abnormal compared to the hippocampus of the conscious rat. It is probably best, therefore, to interpret the results of in vitro experiments as indicating that the tissue is capable of a particular response - as evidence, for example, that a particular pathway exists or that a cell type has receptors for a particular transmitter - rather than as indicating that the observed response actually occurs in vivo.
CHAPTER II. RADIOIMMUNOASSAY EXPERIMENTS

A. METHODS

1. SUMMARY

As described in more detail below, samples of brain tissue from either male Wistar rats or female HoDI rats were homogenized, and the vasopressin was extracted with 0.2 M acetic acid (Wilson and Smith, 1969). The proteins were removed by precipitation with acetone, the lipids removed with petroleum ether, and the vasopressin content of the tissues was determined by radioimmunoassay. All solutions, samples, and containers were kept in ice throughout the homogenization, extraction, and radioimmunoassay procedures. All glassware used for tissue samples or peptide solutions was siliconized.

2. TISSUE SAMPLE PREPARATION

Each rat was anesthetized with urethane (1.0-1.5 g/kg body weight, i.p.) and decapitated. Its brain was immediately removed, rinsed with cold saline, and placed in additional cold saline in a petri dish. The hippocampi were dissected free and divided into approximately equal rostral and caudal portions, and a sample of cerebral cortex of similar size was removed from the midline area above the septum on each side. Right and left portions of each tissue were combined, rinsed with saline, homogenized in 0.2 M acetic acid with a Teflon coated pestle in a Potter-Ehlevehjem assembly, and centrifuged at 15,000 g for 30 minutes. The supernatant was lyophilized and stored at -20°C. Tissues from 6 Wistar
rats were combined to form the "pooled hippocampal tissue" and the "pooled cortical tissue" referred to in the "Results and Analysis" section. The "pooled HoDI cortex" consisted of the entire cerebral cortex caudal to the septum from 3 HoDI rats. Normally the dissecting dish and instruments were rinsed between dissections. One brain from an HoDI rat, however, was deliberately dissected in the saline remaining from the dissection of a Wistar rat brain to determine whether vasopressin leaking from the severed pituitary stalk could be contaminating the tissues during dissection.

3. **ACETONE/PETROLEUM ETHER EXTRACTION**

Vasopressin was extracted from the lyophilized samples by a modification of the method of Robertson et. al. (1973). Each tissue sample was dissolved in 2.0 ml of 2.0 mM acetic acid, and 200 µl was removed for protein determination (Biorad Protein Assay Kit I, with bovine gamma globulin as a standard). Acetone (2.0 ml) was added to the remaining 1.8 ml to precipitate the proteins, and the sample was mixed on a Vortex mixer and centrifuged (1200 g for 50 minutes). After the supernatant was decanted and saved, the pellet was resuspended in 1.0 ml acetone and centrifuged again, the two supernatants were combined, and the pellet was discarded. Lipids were then removed by the addition of 5.0 ml of petroleum ether. The two phases were mixed on a Vortex and allowed to separate for at least one hour before the ether (upper phase) was aspirated and discarded. The thin film at the interface was allowed to remain with the lower phase. After the addition of 100 µl of 0.2 M acetic acid, the sample was evaporated to less than its original 2.0 ml volume under
a stream of nitrogen gas to remove the acetone and any remaining petroleum ether. The sample remained in the ice bath during the evaporation. The final solution was lyophilized and stored at -20°C until assayed.

4. RADIOIMMUNOASSAY

The vasopressin content of the brain tissues was measured by radioimmunoassay. The antiserum ("R-20") was obtained from a DI rat immunized with LVP conjugated to bovine serum albumin (BSA) by the carbodiimide technique (Goodfriend et. al., 1964). The antiserum is specific for vasopressin, arginine vasopressin being about twice as effective as lysine vasopressin, and does not cross react significantly (<1%) with oxytocin, AVT, isotocin, or angiotensin. The vasopressin used as a standard in the assay was a posterior pituitary extract (2.1 IU/ml) kindly provided by Dr. R.E. Weitzman of Harbor General Hospital, Torrance, California. The extract was standardized against synthetic AVP (Spectrum Laboratories) to express the potency on a weight basis.

Iodinated AVP was prepared as described by Weitzman and Fisher (1976), based on the Chloramine-T technique developed by Greenwood et. al. (1963) for iodination of growth hormone. Synthetic AVP (Spectrum, 10 μg in 10 μl of 0.05 M acetic acid) was mixed with 15 μl of 0.5 M phosphate buffer (pH 7.4), 10 μl of Chloramine-T (1.0 mg/ml), and 15 μl Na\textsuperscript{125}I (Amersham, 37 MBq/μl). The reaction was allowed to proceed for 50 seconds at room temperature and was then stopped by addition of 100 μl Pentax BSA (25% in saline). Bio-Rad anion exchanger AG1-X10 (200 μl of a 250 mg/ml suspension) was added to remove unreacted iodine, the mixture was centrifuged briefly, and the supernatant was applied to a column of CM Sephadex
C-25 (9x800mm) and eluted with 0.6 M Na acetate buffer (pH 4.85) at 4°C. The moniodinated hormone had a specific activity of 19-39 MBq per µg of AVP immunoreactivity (1150-2250 DPM/pg iAVP).

All assays were performed in triplicate. The reagents were dissolved in 0.15 M phosphate buffer, pH 7.2, at the concentrations indicated. The reaction mixture consisted of 200 µl of standard or sample, 200 µl of the R-20 antiserum (at 1:12000 dilution), and 400 µl of buffer containing 0.1 M EDTA and 1% rabbit serum. The nonspecific binding (NSB) was measured in tubes containing 200 µl of buffer in place of the antiserum. The assay was incubated at 4°C for 3 days, 200 µl of labelled hormone (3000 DPM) was added to each tube, and the assay was returned to the cold for an additional two days. Bound and unbound ("free") fractions were separated by the addition of 200 µl of dextran-coated charcoal (250 mg of dextran T-70 and 2.5 g of charcoal per 100 ml buffer), followed by centrifugation (1200 g for 10 minutes). Each bound fraction (supernatant) was poured into a separate tube and both bound and free fractions were counted on a Nuclear-Chicago Series 1185 automated gamma counter. The ratio of bound to free radioactivity ("B/F") was calculated with the aid of an Amdahl 470 V/8 computer, and a standard curve, showing the relation between the B/F ratio and the amount of unlabelled hormone, was plotted on semi-logarithmic paper (Figure 3). The sensitivity of the assay, calculated as the amount of hormone required to produce a 20% depression in binding, was 0.24 ± 0.02 pg (mean ± SEM), and the intra-assay co-efficient of variation at 1.0 pg was 3.3% (n=4).

In preliminary experiments, the tissue was assayed using a guinea pig antiserum, GP-15. This antiserum, also produced by immunization with
Figure 3. Standard curve of an AVP radioimmunoassay. The assay was prepared as described in the text, using the R-20 antiserum and a total incubation time of 5 days. The non-specific binding was calculated as the proportion of total radioactivity appearing in the "bound" fraction of tubes containing 200 µl of buffer instead of antiserum (equation 1). As indicated in equation 2, the NSB was subtracted before the B/F ratios were calculated. The terms "B" and "F" are the amounts of radioactivity (as DPM) in the bound and free fractions, respectively, while "b" and "f" refer to the corresponding quantities in the non-specific binding tubes. Values are plotted as means ± SEM, n=3.
(1) \[ (NSB) = \frac{b}{b + f} \]

(2) \[ \frac{B}{F} = \frac{B - (NSB)(B + F)}{F} \]
LVP coupled to BSA, does not distinguish between LVP and AVP but shows less than 0.5% cross-reaction with oxytocin, isotocin, and angiotensin. The GP-15 antiserum was also used for a variety of other purposes such as checking solutions and measuring the amount of vasopressin ejected from iontophoretic micropipettes. The assay was similar to the R-20 assay except that the EDTA was omitted, BSA (25 mg/100 ml) was sometimes substituted for the rabbit serum, and the total incubation period was occasionally reduced to 3 days.

B. VASOPRESSIN CONTENT OF RAT BRAIN TISSUES

1. RESULTS AND ANALYSIS

The amounts of vasopressin in the hippocampus and the cerebral cortex of 9 male Wistar rats (220-430 g) and 7 female HoDI rats (182-250 g) were determined by radioimmunoassay and are summarized in Table IV. Vasopressin was detected in all 9 of the ventral hippocampal samples and in all but one of the 9 dorsal hippocampal samples from the Wistar rats. The amount of vasopressin in the ventral hippocampus (81.4 ± 8.3 pg iAVP/mg soluble protein) was 2.7 times greater than in the dorsal hippocampus (30.3 ± 7.5 pg iAVP/mg soluble protein). In contrast to this, none of the cortical samples from the Wistar rats contained detectable vasopressin (<6.2 pg iAVP/mg soluble protein). No immunoreactivity was detected in any of the tissue samples from the HoDI rats, confirming that the immunoreactive material in the Wistar rats was in fact AVP.

The recovery of 11.25 pg AVP added to each of 4 HoDI cortical samples and 2 Wistar cortical samples was 45.7% ± 4.18% (mean ± SEM, n=6), and the values in Table IV have therefore been corrected appropriately. Since
TABLE IV. VASOPRESSIN CONTENT OF RAT BRAIN TISSUES

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>n</th>
<th>iAVP (pg/mg soluble protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HoDI V. HIPP.</td>
<td>6</td>
<td>n.d.</td>
</tr>
<tr>
<td>HoDI D. HIPP.</td>
<td>5</td>
<td>n.d.</td>
</tr>
<tr>
<td>HoDI CORTEX</td>
<td>2</td>
<td>n.d.</td>
</tr>
<tr>
<td>WISTAR V. HIPP.</td>
<td>9</td>
<td>81.4 ± 8.3</td>
</tr>
<tr>
<td>WISTAR D. HIPP.</td>
<td>9</td>
<td>30.3 ± 7.3</td>
</tr>
<tr>
<td>WISTAR CORTEX</td>
<td>7</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

All values have been corrected for extraction loss as described in the text. The values are means ± SEM. AVP was not detected (n.d.) in the Wistar cortex or in the HoDI tissues. The hippocampal tissue was divided into ventral (V. HIPP.) and dorsal (D. HIPP.) portions. Each sample represents the tissue from one rat.

The AVP was added to the tissue immediately after dissection, the correction factor should compensate for loss of hormone during all stages of tissue processing and for the effect of nonspecific interference.

To determine whether the vasopressin found in the Wistar hippocampi could have resulted from contamination of the tissue during dissection (by peptide released, for example, from the severed pituitary stalk), an HoDI rat brain (the "contamination control") was dissected in saline previously used for dissection of a Wistar rat brain. The dissection was deliberately performed in a manner intended to maximize the chance of contamination. The tissue samples from this brain did not contain detectable vasopressin. This finding, and the absence of vasopressin in the Wistar cortical samples, indicate that contamination could not have accounted for the vasopressin detected in the Wistar hippocampal tissues.
To allow larger amounts of material to be assayed, brain tissue from several rats was combined ("pooled tissues"). Pooled hippocampal tissue prepared from 6 male Wistar rats (410-480 g) contained 236 pg iAVP/mg soluble protein (value corrected as described above), 2.9 times as much as the single ventral hippocampal samples, and pooled cortical tissue from the same rats contained 26.3 pg iAVP/mg soluble protein, even though iAVP was not detectable in the single cortical samples. Pooled cortical tissue from 3 HoDI rats (185-215 g) failed to yield detectable iAVP. The curve produced by serial dilution of the pooled tissue was parallel to that produced by serial dilution of the AVP standard (Figure 4), as would be expected if the immunoreactive material in the tissue was immunologically identical to AVP.

2. DISCUSSION

Immunocytochemical studies in the rat (Buijs et. al., 1978; Buijs and Swaab, 1979; Buijs, 1980; Sofroniew and Weindl, 1981) have demonstrated that hypothalamic neurones containing vasopressin project to the hippocampus. The present study confirms the presence of iAVP in the rat hippocampus and suggests that the ventral hippocampus contains more of this peptide than does the dorsal portion. This finding is consistent with the report by Buijs (1980) that the immunoreactive fibers project primarily to the ventral hippocampus, but it is not in agreement with the work of Sofroniew and Weindl (1981), who found the dorsal hippocampus to be devoid of vasopressin containing fibers. The difference between these two immunocytochemical studies has been discussed more fully in Chapter I of this thesis.
Figure 4. Serial dilution of hippocampal tissue. The curves produced by serial dilution of the AVP standard (open circles) and of the pooled Wistar hippocampal tissue (closed circles) are shown. The abscissa for the tissue curve is expressed in µg soluble protein/assay tube. The relation between the two abscissae is arbitrary and was chosen to facilitate comparison. Values are plotted as means ± SEM, n=3.
The cerebral cortex, included in the study as a control, did not contain detectable vasopressin. While immunoreactive fibers have been found in some cortical areas, such as the entorhinal region, the tissue included in the present study was taken from the dorsal surface of the cortex, where immunoreactive fibers have not been reported.

George and Jacobowitz (1975) measured the AVP content of 32 areas of the Sprague-Dawley rat brain by radioimmunoassay. They were unable to detect any AVP in the hippocampus or in any other extrahypothalamic tissue, including areas such as the habenula and the organ vasculosum of the lamina terminalis (OVLT) which have been reported to contain immunoreactive fibers (Buijs et. al., 1978; Buijs, 1980; Sofroniew and Weindl, 1978). This failure, however, is probably attributable to the lack of sensitivity of their assay (100 pg/mg protein).

Using a more sensitive assay system, Dogterom et. al. (1978) found immunoreactive vasopressin in a variety of extrahypothalamic areas (hippocampus, cortex, septum, OVLT, amygdala, nucleus parafascicularis, choroid plexus, and medulla) of the Wistar rat brain. The anterior hippocampus contained 58 ± 37 pg iAVP/mg dry weight, the posterior hippocampus contained 30 ± 11 pg iAVP/mg dry weight, and the cortex contained 4.6 ± 1.2 pg iAVP/mg dry weight. No iAVP was found in any HoDI tissues. In their study, in which the samples were "dissected or punched out" of freeze dried brain sections, the "anterior" hippocampus included only the anterior tip of the hippocampus and the "posterior" hippocampus extended only as far caudally as plane A2420 (König and Klippel, 1967). Most of the tissue referred to as "ventral hippocampus" in the present study was not included in their study. The amount of vasopressin detected
in the hippocampus in the present study would correspond to approximately 2 pg iAVP/mg dry weight, computed for the hippocampus as a whole, and was therefore considerably less than reported in their study. The discrepancy may be partly the result of differences in dissection technique. In the present study, the fimbria was removed and only the vasopressin in the hippocampus itself was measured. Since the immunoreactive fibers run through the fimbria to reach the hippocampus (Buijs, 1980), the concentration of vasopressin may be higher in the fimbria than in the hippocampus. The report by Dogterom and his coworkers does not mention whether the fimbria was removed.

In a study by Hawthorn et. al. (1980), the rat hippocampus was found to contain 27 pg iAVP/mg total protein, which was about 4 times greater than reported in the present study (approximately 6.7 pg iAVP/mg total protein). Vasopressin was also reported in such extrahypothalamic areas as the thalamus, amygdala, and cerebellum, and small amounts were present in some cortical areas. No HoDI controls were used, but thin layer chromatography of the hippocampal tissue suggested that the immunoreactive material was in fact AVP. As a further check, the curves produced by serial dilution of 3 of the brain tissues (not including the hippocampus) were compared to the standard curve; whether the curves were parallel, as claimed by the authors, is debatable.

The finding of immunoreactive AVP in the rat hippocampus supports the immunocytochemical demonstration of immunoreactive fibers in the hippocampus. The evidence would seem to be sufficient to justify neurophysiological studies on the action of AVP and related peptides on hippocampal neurones. Such studies form the basis of the remainder of this thesis.
CHAPTER III. NEUROPHYSIOLOGICAL EXPERIMENTS

A. METHODS

1. IN VITRO STUDIES

Hippocampal slices were prepared from either male Wistar rats (160-360 g) or female HoDI rats (180-190 g). The peptides to be studied were added to the perfusion liquid, and the responses of individual neurones were recorded extracellularly with single barrel glass micro-pipettes.

Slice Preparation

Each rat was decapitated and its brain was placed in oxygenated (95% O$_2$, 5% CO$_2$) Krebs solution in a petri dish. One hippocampus was dissected free, using Teflon coated spatulas, and cut into 400 μm transverse slices, approximately in the plane of the hippocampal lamella (Skrede and Westgaard, 1971), on a McIlwain tissue chopper. The slices were immediately transferred to a nylon net and placed in a narrow, rectangular incubation chamber in a block of acrylic (Figure 5). The lower surface of each slice was in contact with a thin film of oxygenated Krebs solution that flowed along the bottom of the chamber at a constant rate (about 0.5 ml/minute), and the upper surface was exposed to the humidified O$_2$/CO$_2$ mixture. The chamber was contained in a water bath maintained at a constant temperature. In most experiments a temperature of 33°C was chosen as a compromise between the more rapid deterioration of the slices that occurred at higher temperatures and the poor responsiveness observed with lower temperatures.
Figure 5. Schematic diagrams of the incubation chamber. A nylon net is glued to an acrylic frame, which is then placed in a narrow groove in an acrylic block. The flow of the Krebs solution is indicated by the broad arrows. The net rests on the bottom of the chamber, and the Krebs solution must therefore flow through the mesh of the net, passing along the underside of the slice. The solution then flows under the edge of the frame into a second chamber, where it is removed by an aspirator. An acrylic divider, shown in cut-away view, prevents the aspirator from drawing air over the slice.
The apparatus used for most of the in vitro experiments is shown schematically in Figure 6. Oxygenated Krebs solution stored in the barrels of 50 ml plastic syringes was allowed to flow by gravity through Tygon tubing (I.D. 0.06 inch) to a drip chamber (a 5 ml plastic syringe) and then through Silastic tubing to the recording chamber. The Tygon tubing could be clamped, and solution was allowed to flow from only one of the syringes at a time. Peptides were applied by interrupting the flow of control solution and simultaneously removing the clamp on the tubing from a reservoir of solution containing the peptide. The flow rate could be controlled by an adjustable clamp below the drip chamber. The rate was monitored by timing the drops in the drip chamber with a stopwatch, previous measurements having established that the volume of the drops did not differ by more than 10%. The relative heights of the individual syringes were adjusted as needed to ensure that the flow rate was not affected by changing from one solution to another. The design of the apparatus used in some of the experiments was slightly different from that illustrated in Figure 6. There were differences, for example, in the types and sizes of containers used for the Krebs solutions, in the design of the frame used to hold the nylon net, and in the method of channeling the solution from the net to the aspirator. Separate drip chambers were sometimes used for each source of solution to reduce the lag time when changing solutions. The separate chambers were joined by a 5-way connector to a single Silastic tube. A more elaborate arrangement, involving 6 syringes and 2 drip chambers, was used in several experiments to allow peptides and antagonists to be applied simultaneously.

The Krebs solution contained 124 mM NaCl, 5.0 mM KCl, 26.0 mM NaHCO₃,
Figure 6. Apparatus for maintaining hippocampal slices *in vitro*. The electrode is positioned in the slice (not shown), which rests on a nylon net in an acrylic chamber (see Figure 5). The chamber is placed in a heated water bath. The Krebs solution is allowed to flow by gravity from 50 ml syringes. The heights of the syringes can be individually adjusted to provide a uniform flow rate.
1.25 mM NaHPO₄, 2.0 mM MgSO₄, 10.0 mM glucose, and 2.0 mM CaCl₂. Solutions containing lower concentrations of CaCl₂ (1.0 or 1.5 mM) were also tested, and the amount of NaCl was adjusted accordingly to maintain isotonicity. In some experiments the peptides were simply dissolved directly in the Krebs solution. In others, the lyophilized peptides were first dissolved in 20-100 μl of acid (1.0 mM HCl in 140 mM NaCl). The acid solution was then diluted 500-1000 fold with Krebs solution and neutralized with 20-100 μl of NaOH (1.0 mM NaOH in 140 mM NaCl). All control and peptide solutions were acidified and neutralized in the same way in any given experiment. The peptides included AVP (Sigma, grade VIII), LVP (Sigma, grade IV), Oxytocin (Sigma), ddOT (Beckman), ddAVP (Beckman), PLG (Beckman), and somatostatin (Serono). Dr. M. Manning of the Medical College of Ohio, Toledo, Ohio, kindly provided AVP, DGAVP, Gly⁷-OT, d(CH₂)₅Tyr(Me)AVP, and d(CH₂)₅-D-Tyr(Me)VAVP.

Recording Techniques

The response to the peptides was monitored by extracellular unit recording. Single barrel glass micropipettes filled with 4 M NaCl and having an impedance of 0.5-4.0 MΩ were lowered into the cell body layer with a manually operated micromanipulator. Accurate positioning of the electrode was facilitated with a stereoscopic microscope. The signal from the microelectrode was passed to a preamplifier, and the amplified, compensated signal was then sent to a differential amplifier for further amplification and filtering (0.1-3 kHz or 1-3 kHz) and was displayed on an oscilloscope. Several combinations of Tektronix amplifiers and oscilloscopes were used. A platinum wire running the length of the incubation
chamber served as the reference electrode. (Silver wires were found to tarnish in the presence of sulfur containing peptides.) The filtered signal was also conducted to an EKEG Neuroratemeter or a Winston discriminator. Spikes above a predetermined amplitude activated a Schmidt trigger, and the output from the trigger was displayed simultaneously with the filtered signal on a dual beam storage oscilloscope. The trigger level was adjusted to prevent its being activated by background noise, and a second output from the trigger was recorded, as spikes per second, on an Esterline-Angus chart recorder. The condition of the slices was judged by both visual (visibility of the cell line, texture of the surface) and electrophysiological criteria (Teyler, 1980). Evoked potentials were studied by positioning a bipolar stimulating electrode (twisted Nichrome wire) in the Schaffer collateral pathway and applying stimuli of 1.0-1.5 msec duration and usually of 10-30 volt amplitude either with a Digitimer D100 timer and a Digitimer isolated stimulator model DS-2 or with a Grass stimulator S4KR and a Grass stimulus isolation unit SIU5. The evoked potentials were recorded through the same microelectrode used for single unit recording but with a low-pass filter of 1 Hz.

2. **IN VIVO STUDIES**

The effect of vasopressin and oxytocin on neurones in the lateral septum and the hippocampus was studied *in vivo* in 24 male albino rats (240-485 g). The peptides were applied by microiontophoresis, and the response of the neurones was monitored by extracellular unit recording.
Micropipettes

Multibarrel micropipettes, consisting of 7 fused glass capillary tubes were purchased from Vancouver Scientific Glassblowing and were drawn to a fine tip on a vertical microelectrode puller. The tip was broken back to 5-12 μm, and the micropipette was boiled until all barrels were filled with water. Most of the water was then withdrawn from each barrel and replaced either with 4 M NaCl for recording (central barrel) or with the appropriate solution (glutamate 0.5 M, pH 8.6; acetylcholine bromide 1 M, pH 4.9, or the peptide). The micropipette, with its tip immersed in water, was then refrigerated for at least 24 hours to allow the solutions to diffuse into the tip. The peptides tested were arginine vasopressin (Sigma grade VIII, Spectrum), lysine vasopressin (Sigma Grade IV), and oxytocin (Sigma). All of these are supplied as a lyophilized powder and were dissolved in dilute acetic acid for use in this experiment. Sigma AVP grade VI, which is supplied as a 0.4 mM solution containing 0.33 mg NaCl and 0.03 mg NH₄Cl per ml, and Sandoz oxytocin (Syntocinon-10) which contains chlorobutanol (5 mg/ml), were used less frequently. All of these peptides had a pronounced tendency to block the barrels of the micropipette. In an effort to circumvent this problem, the peptides were tested at many different concentrations (10⁻⁶ to 5×10⁻⁴M) in various concentrations of acetic acid (10⁻⁵ to 2×10⁻²M). The more dilute solutions contained 10-40 mM NaCl in addition to the peptide and acetic acid. The ability of the micropipettes to eject AVP was determined by immersing the tip of the micropipettes and a silver ground wire into 1.0 ml saline, applying the appropriate currents (+20 to +60 nA) for 30 to 60 minutes, and measuring the AVP content of the saline by radioimmunoassay. The
measured rate of ejection of AVP was compared to the theoretical rate of release, which was calculated assuming a charge of +2 for the AVP molecule and apportioning the total current according to the relative concentrations and molecular weights of all positively charged substances present. The rates of ejection from each of 6 freshly prepared single barrel micropipettes containing 3 different AVP solutions were 0.015-2.0 pg/nA/min, compared to theoretical rates of 2.0-4.2 pg/nA/min. The rates of release from each of 5 barrels of 2 multibarrel micropipettes were below the limit of detection (< 0.006 pg/nA/min, compared to theoretical rates of 0.09-11.8 pg/nA/min). These two micropipettes had been previously used in experiments and had been prone to blockage, as described in the "Results and Analysis" section.

Surgery

Each rat was anesthetized with urethane (1.5 g/kg, ip), its head was shaved, and it was placed in a Kopf stereotaxic holder with the incisor bar 4.2 mm below the mid-aural line. The skin over the skull was cut along the midline with a scalpel, and the skull was cleared with a periosteal elevator and allowed to dry. The skull over the area to be studied was opened with a dental drill, the flap of skull was removed with forceps, and the dura was carefully cut away. Particular care was taken when removing the skull and dura over the septum to avoid damage to the superior sagittal sinus. Bleeding from bone was controlled, when necessary, with bone wax, and bleeding from small vessels was stopped with Stroll's hydrocellulose wedges or Upjohn gel foam. The exposed brain tissue was kept moist with warm Locke's solution (37°C). When surgery was complete, the skin was raised and sutured to an acrylic holder.
The resulting pocket was filled with Locke's solution which was then covered with a layer of mineral oil.

Recording and Iontophoresis

The equipment and procedures for stimulation and for recording single units and evoked responses were essentially the same as those used for the in vitro experiments, except as described below. The central barrel of the 7 barrel micropipette served as the recording electrode (0.4-2.0 MΩ), and a concentric steel electrode with a tip separation of 0.5 mm was used for stimulation. The reference electrode was a wire clipped to the edge of the surgical incision. Currents for iontophoresis were controlled with potentiometers and were passed through 1000 MΩ series resistors before being conducted by silver wires into the barrels of the micro pipettes. A panel of lights indicated the amount of current actually passing through the circuit and therefore showed whether the barrel was blocked, retaining currents of 5-15 nA and of opposite polarity to the ejecting currents prevented diffusion from the tip of the micropipette (Purves, 1979; Clarke et. al., 1973; Kelly et. al., 1975).

The recording electrode was lowered into the brain in 4 μm steps using an electronically controlled microdrive mounted on a large micro-manipulator. The coordinates for electrode placement were determined from an atlas of the rat brain by König and Klippel (1967) except that measurements were made from bregma, corresponding to their plane A7190 μm, instead of from the inter-aural line. The coordinates below are given as the distance in millimeters anterior (A), posterior (P), or lateral (L), to bregma, or ventral (V) to the surface of the cortex. Recordings
were made in the lateral septum in the area from bregma to 0.9 mm anterior
to bregma. The lateral and ventral coordinates depended on the anterior-
posterior position but were typically in the range of 0.8-1.2 L and 3.0-
5.0 V. The stimulating electrode was usually placed in the ipsilateral
hippocampus at 3.5 P, 2.5 L, 2.5 V. Recordings were also made in the hipp-
campus (4.0-5.2 P, 2.0-3.5 L), and in this case the depth of the electrode
was determined by locating the evoked response in the dentate gyrus
(Lømo, 1971a) following stimulation of the ipsilateral entorhinal cortex
(8.1 P, 4.3 L, 2.5 V). At the end of the experiment, an 18 μA anodal cur-
rent was passed through the stimulating electrode for 20-30 seconds. The
brain was then fixed in formalin containing 1% potassium ferrocyanide,
frozen, sectioned, and stained, and the Prussian blue spot marking the
position of the iron deposit was located under the microscope. Less fre-
quently, the position of the recording electrode was determined by ejecting
pontamine sky blue (4% in 1M NaCl) from one of the outer barrels of the
micropipette (12 μA X 10 minutes).

B. IONTOPHORETIC APPLICATION OF NEUROHYPOPHYSIAL
PEPTIDES

1. RESULTS AND ANALYSIS

The effect of neurohypophysial peptides applied by iontophoresis to
cells in the lateral septum or the hippocampus was studied in 24 male
Wistar rats (240-485 g). Iontophoresis of vasopressin (AVP or LVP) was
accompanied by a gradual increase in the firing rate in 19 of 100 cells
in the lateral septum and in 20 of 45 cells in the pyramidal cell layer
of the hippocampus (Figures 7 and 8). None of the 4 cells tested in the granule cell layer of the dentate gyrus were excited by vasopressin. There was no obvious difference between the effect of vasopressin on cells firing spontaneously and on those excited by continuous application of glutamate or acetylcholine. Depressions of spontaneous or drug-induced firing were observed less frequently than excitations and were characterized by a sudden onset and offset (Figure 9). A few cells responded with both a sudden inhibition and a gradual excitation (Figure 10), and sudden excitations were observed in two cells.

Iontophoresis of oxytocin was attempted only in the septum. Excitations were never observed during iontophoresis of oxytocin, although sudden inhibitions were observed with 7 of the 30 cells tested.

Iontophoresis of either peptide often resulted in gradual blockage of the micropipette barrel — that is, in an increase in the resistance of the barrel so that only small currents could be passed. Tests on 5 partially blocked micropipette barrels revealed that none could expel detectable AVP (limit of detection was 0.006 pg/nA/min; see Chapter III, section A2). In contrast, AVP release from unblocked micropipettes was in the range 0.015-2.0 pg/nA/min (n=6). For reasons discussed in Chapter I, section C2, one would not expect significant peptide release from blocked or partially blocked micropipettes, the current being carried instead by H⁺ or other small ions. Micropipette barrels containing oxytocin were blocked much more frequently than barrels containing the more highly charged molecules AVP or LVP.

Repeated applications of vasopressin sometimes resulted in a decline in the responsiveness of the cell (Figure 11). In some cases this decline
Figure 7. Response of neurones in the hippocampus to iontophoresis of AVP. In this and all subsequent ratemeter records, the firing rate is measured on the ordinate in spikes per second and time on the abscissa in minutes. The iontophoretic currents were applied for the period of time represented by the horizontal bars; the numbers over the bars indicate the magnitude of the current in nanoamperes. This figure illustrates the response of three neurones in the hippocampus to AVP. Note, in trace B, the lack of response to iontophoresis of H⁺ ions from a dilute solution of acetic acid.
Figure 8. Response of neurones in the septum to iontophoresis of AVP.

The responses of 3 cells are shown.
Figure 9. Inhibition of neurones in the septum by iontophoresic currents. The activity of the neurone represented in trace A was the result of continuous application of glutamate (25nA) during the period of the recording. The cell in trace B was spontaneously active.
Figure 10. Neurone responding with an inhibition followed by an excitation. In trace A, glutamate (4 nA) was applied continuously; in trace B, from the same hippocampal neurone, no glutamate was applied.
Figure 11. Decline in responsiveness during repeated applications of AVP. The responses of a single hippocampal neurone to repeated applications of AVP are shown. The three traces are continuous except as indicated.
may have been due to progressive blockage of the micropipette tip, as evidenced by the increasing resistance of the barrel, rather than to tachyphylaxis. The sudden inhibitions, on the other hand, were not altered by changes in resistance and were probably due to non-specific current effects.

Control solutions, consisting of dilute solutions of acetic acid identical to those used to dissolve the peptides, were tested on most cells that responded to vasopressin. Of the 39 cells excited by AVP or LVP, 6 were also excited by the control solution (Figure 12). The response to the control solution would seem to suggest that the excitations could have been caused by H\(^+\) ions rather than by vasopressin, but this was not the case: the excitations were never observed during iontophoresis of oxytocin, even though most of the current from the oxytocin barrels would be carried by H\(^+\) ions. Assay of the acetic acid solutions from two micropipettes revealed that the solutions were contaminated with AVP (3\(\times\)10\(^{-9}\) M and 5\(\times\)10\(^{-9}\) M, compared to 4\(\times\)10\(^{-9}\) M AVP in the vasopressin barrel). Contamination to this extent is probably unavoidable; the micropipettes were stored in a refrigerator with the tips immersed in water and were therefore coated with a continuous film of condensed water, which would allow diffusion between the upper ends of the barrels. Some of the oxytocin barrels might also have been contaminated by vasopressin. The vasopressin would not have been expelled as readily from the oxytocin barrels as from the control barrels, however, since the oxytocin barrels almost invariably exhibited some degree of blockage and would therefore have passed much less peptide for a given current.

The response to iontophoretic application of vasopressin was also
Figure 12. Excitation produced during iontophoresis of $\text{H}^+$ ions. The two hippocampal neurones responded consistently to both $\text{H}^+$ and AVP.
studied in hippocampal slice preparations from 8 Wistar rats. Gradual excitations were observed with 5 of the 14 cells tested (Figure 13). The response did not differ from those reported for the in vivo studies.

The results presented here indicate that AVP and LVP can cause relatively rapid changes in the activity of neurones in the septum and the hippocampus. Neurones in the septum did not seem to respond to oxytocin, but it is possible that the oxytocin was not expelled from the micro-pipettes in sufficient quantities. Oxytocin was not tested in the hippocampus. Since the response to vasopressin occurred even in vitro, it is not likely to have been the result of vasoconstriction. Furthermore, Lassoff and Altura (1980) have reported that vasopressin does not cause constriction of the cerebral microvasculature.

2. DISCUSSION

Nerve terminals containing immunoreactive vasopressin have been demonstrated at both the light and electron microscopic level in the lateral septum and the hippocampus of the rat (Sofroniew and Weindl, 1978; Buijs and Swaab, 1979; Buijs, 1980). The work reported here indicates that neurones in these areas respond to vasopressin applied by iontophoresis and therefore suggests that vasopressin is, in fact, the active principle, or at least one of the active principles, released by the fibers.

That vasopressin can affect the firing rates of neurones is not, in itself, new. Nicoll and Barker (1971) found that 80% of neurones in the cat supraoptic nucleus (SON) were inhibited by iontophoresis of LVP. A few excitatory responses were also observed. The opposite response was
Figure 13. Response to iontophoresis of AVP in vitro. The effect of AVP on two neurones in the in vitro hippocampus is illustrated. The AVP was applied by iontophoresis in the cell body layer of the CA1 region.
observed in the cortex, where 90% of neurones were excited by LVP. The sensitivity of cortical neurones is somewhat surprising, since there is no evidence that fibers containing vasopressin project to this area. In a similar study by Moss et. al. (1972), oxytocin applied by iontophoresis was found to excite a majority of neurosecretory cells in the paraventricular nucleus (PVN) of the rat and rabbit. The peptide had no effect on non-neurosecretory cells in the PVN - that is, on cells which were not antidromically invaded following stimulation of the neural lobe - or on cells in the SON, cortex, or thalamus. Very low concentrations of peptide (10^{-7} M) and weak currents (0.5-30 nA) were used, and the responses were relatively small; the authors report that the use of higher concentrations of oxytocin in the micropipette caused such "massive excitation" that the cells were "lost". Vasopressin (AVP) applied in the same way had little effect on SON (one cell excited, one inhibited, 3 unaffected) or PVN cells (2 of 29 PVN cells were inhibited). The sensitivity of neurosecretory cells to neurohypophysial peptides seems to extend even to invertebrate species. Vasopressin or oxytocin applied either in the bathing medium (threshold 10^{-9} M) or by iontophoresis caused long lasting excitation of neurosecretory cells of the snail *Aplysia californica* (Barker et. al., 1975). The neurosecretory cells are thought to release a peptide similar to the neurohypophysial peptides (Barker, 1976). As discussed more fully in the introduction to this thesis, however, there has been relatively little direct evidence that neurones in putative target areas outside the hypothalamus respond to the NHP peptides. Morris et. al. (1980) showed that oxytocin, applied by iontophoresis or pressure ejection, depressed the activity of neurones in the rat caudal
medulla, but there was no close correlation between the location of the responsive cells and the areas where immunoreactive fibers were found.

The present study demonstrates that the activity of neurones in two areas rostral to the hypothalamus can be altered by local application of vasopressin. Both of these areas, the septum and the hippocampus, have previously been suggested as possible target areas of the NHP peptides. Workers in Calgary (Kasting et. al., 1979), for example, have shown that vasopressin has an antipyretic effect when injected into the septum of sheep. Because of the difference in species and technique, it is not clear whether the AVP sensitive neurones studied in the present experiment correspond to those involved in antipyresis. Kovács et. al. (1979b) have reported that vasopressin facilitated passive avoidance behavior when injected into the dorsal septal nuclei or the medial septal nuclei of the rat. In contrast to the present study however, oxytocin was found to be equally effective. This discrepancy may reflect the difference in location (dorsal and medial portions of the septum versus the lateral septum). The demonstration that iontophoresis of AVP and LVP can alter the activity of hippocampal neurones would seem to be in agreement with the report by Huston et. al. (1977) that injection of 10 μM LVP into the hippocampus of anesthetized rats resulted in hippocampal spreading depression. The difference between the excitations observed with iontophoresis and the depression that occurred with the intrahippocampal injections do not represent a conflict, since spreading depression can result from the massive depolarization induced by an excitatory agent. In the same study, for example, surface application of the depolarizing agent KCl onto the cortex caused cortical spreading depression. The role of the NHP peptides
in hippocampal function will be explored more fully in the next section of this thesis.

While the work reported here indicates that vasopressin excites neurones in the lateral septum and the hippocampus of the rat, iontophoresis was found to be a less than ideal technique for studying peptide induced excitations. The problem of contamination of the control barrels could probably be solved by the use of lower concentrations of the peptides. Most workers seem to use quite high concentrations of peptide for iontophoresis (Yarbrough and Haubrich, 1978; Felix and Phillips, 1979; Duggan et. al., 1979), but the work by Moss et. al. (1972) and by Barker et. al. (1975), described above, indicates that much lower concentrations would have been effective. The tendency of the peptides to block the micropipette barrel was a more serious problem. Further study of the peptides would require the use of antagonists, which are more lipophilic than oxytocin and vasopressin and would therefore be even more difficult to expel from the micropipette. Palmer et. al. (1980) have suggested that micropressure ejection is a more suitable technique than iontophoresis for the study of peptides. Alternatively, one can apply the peptides via the bath solution in an in vitro preparation. This latter approach was chosen and is the basis for the remainder of this report.
C. IN VITRO EXPERIMENTS

1. RESULTS AND ANALYSIS

The NHP peptides were found to have a pronounced, dose dependent excitatory effect when applied in concentrations of $10^{-9}$ to $10^{-6}$ M onto rat hippocampal slices. An example of the excitatory action of 0.5 μM AVP can be seen in Figure 14. Not all cells responded to the peptides (see below), and many cells that appeared to respond were rejected for further study for technical reasons such as small spike amplitude or excessive background noise. The data below are based on a study of 57 cells that responded unambiguously to repeated applications of peptide.

The effects of the other 3 naturally occurring NHP peptides - LVP, AVT, and oxytocin - were qualitatively and quantitatively similar to that of AVP (Figure 15). Most neurones were tested with more than one of these peptides, and in all cases responded either to all of the peptides tested ("peptide sensitive cells") or to none of them. No cell was found which responded to one NHP peptide but failed to respond to another. This was not true of the peptide somatostatin (SST): while one cell responded to both SST and oxytocin, two cells sensitive to the NHP peptides failed to respond to SST, and one cell insensitive to AVP was excited by SST (Figure 16).

Several synthetic analogues of the NHP peptides were also tested (Table IV). The oxytocin fragment PLG was found to be totally inactive in tests on 4 cells sensitive to oxytocin and to AVT. The ratemeter record of a cell that was unaffected by $10^{-5}$ M PLG but which responded to oxytocin at concentrations as low as $10^{-9}$ M is shown in Figures 17 and
Figure 14. Effect of superfusion of AVP on hippocampal neurones in vitro. The superfusing solution was allowed to flow around the hippocampal slices continuously. During the period represented by the horizontal bar, a solution containing 0.5 μM AVP was substituted for the control solution. Both neurones shown in this figure were in the same slice.
Figure 15. Effect of LVP, AVT, and OXT on hippocampal neurones in vitro. The responses in trace A and trace B were from the same cell.
Figure 16. The effect of SST on a neurone insensitive to AVP. Note the discontinuity in the time scale.
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<td>d(CH₂)₅Tyr(Me)AVP</td>
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<td>d(CH₂)₅-D-Tyr(Me)VAVP</td>
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Table V. Activity of Neurohypophysial Peptides and Analogues in Hippocampal Slices.
Gly⁷OT, which is active at oxytocin (OT or ME) receptors but not at vasopressin (VP or AD) receptors (Lowbridge et al., 1977), was tested on 4 peptide sensitive cells and was found to be as effective as oxytocin. In contrast, DGAVP, tested on two cells sensitive to AVP, produced only a very weak excitation of one cell (Figure 19) and failed to excite the other. The oxytocin analogue desamino-dicarba-oxytocin (ddOT), which is protected against enzymatic attack at the N-terminus and at the disulfide bond, was found to be an effective agonist when tested on 6 peptide sensitive cells. The activity of the analogue was slightly greater than that of oxytocin, suggesting that oxytocin may be partially inactivated by aminopeptidase activity in the hippocampal slice. The equivalent AVP analogue, ddAVP, was also active (1 cell), but its activity was not compared with that of AVP.

The response to all of the NHP peptides could be blocked by prior application of the vasopressin antagonist d(CH₂)₅Tyr(Me)AVP. The specificity of the antagonist is apparent in Figure 20; prior application of antagonist at a concentration of 0.1 μM almost completely blocked the response to 0.4 μM ddOT but had no effect on the response to 2 mM acetylcholine. The antagonist was also tested against AVP, LVP, AVT, and oxytocin and was effective against each of these peptides. In some experiments, the antagonist was dissolved in 1% acetic acid before being diluted 5000 fold in Krebs solution. Control solutions containing the same amount of acetic acid had no detectable effect on the responsiveness of the cells. A second vasopressin antagonist, d(CH₂)₅-D-Tyr(Me)AVVP, was tested against AVP, oxytocin, ddOT, and Gly OT. Like d(CH₂)₅Tyr(Me)AVP, this antagonist consistently blocked the effect of the NHP peptides but
Figure 17. Failure of a hippocampal neurone to respond to PLG. The 3 tracings are from a single cell which responded consistently to AVT and to OXT but did not respond to PLG.
Figure 18. Response of a hippocampal neurone to low concentrations of OXT. Both tracings are from the same cell shown in Figure 17, which failed to respond to 10 μM PLG. The response to $10^{-9}$M OXT was delayed for 12 minutes.
Figure 19. Effect of DGAVP on hippocampal neurones. (A) DGAVP was found to produce a weak excitation of one cell but (B) failed to excite a second peptide sensitive cell from the same rat. (C) Stimulation of the Schaffer collateral pathway resulted in an inhibition of the Gly7OT induced activity of the cell in trace B. Two sweeps were superimposed. (D) The population spike produced by stimulation of the same pathway (30 Volts, 0.2 msec).
Figure 20. Antagonism of the response to ddOT by d(CH$_2$)$_5$Tyr(Me)AVP. The three tracings are from the same cell, before (A), during (B), and after (C) exposure to the antagonist ("ANTAG"). Application of the antagonist is indicated by the dotted line. The antagonist was applied for 10 minutes before the start of trace B, and had been discontinued 20 minutes prior to the start of trace C. The response to ddOT, but not that to acetylcholine (Ach), was almost completely blocked by the antagonist. In C, the cell is beginning to recover from the effects of the antagonist.
not that of acetylcholine (Figure 21). The two antagonists were tested on a total of 15 cells and never failed to block the response to any of the peptides.

The changes in firing rate produced by application of the peptides cannot be attributed to mechanical disturbance of the slice, since changing from one control solution to another never produced a detectable change in the firing rate of the cells. Similarly, changes in the flow rate of the superfusing solution were usually found to be without effect, even when the rate was more than doubled. On one occasion, however, a 17% increase in flow rate was followed by a slight excitation. Total cessation of the flow produced a pronounced excitation of one cell.

Repeated brief applications of peptide by superfusion did not cause peptide sensitive cells to become less responsive, provided that the firing rate of the cell was allowed to return to basal levels between applications. When subjected to continuous application of peptide however, the cells became unresponsive fairly quickly. As can be seen in Figure 22, a cell rendered unresponsive by continuous superfusion with oxytocin appeared thereafter to be insensitive to AVP as well, a finding that suggests that both peptides act on the same receptor.

There were few spontaneously active cells in the slice preparations, but 7 of 8 spontaneously active cells (88%) were peptide sensitive. Most of the peptide sensitive cells included in this study had little or no spontaneous activity but could be induced to fire by superfusion with peptide - the cells were discovered by exposing the slices to one of the peptides and then searching for active cells. The peptide sensitive cells were located in the pyramidal cell layer of area CA1. Stimulation
Figure 21. Antagonism of the response to Gly\textsuperscript{7}OT by d(CH\textsubscript{2})\textsubscript{5}-D-Tyr(Me)VAVP. The antagonist d(CH\textsubscript{2})\textsubscript{5}-D-Tyr(Me)VAVP ("ANTAG") completely blocked the response to Gly\textsuperscript{7}OT but not the response to Ach. Both traces are from the same cell, and the time scale is continuous except as indicated.
Figure 22. Decline in responsiveness of a cell subjected to continuous exposure to oxytocin. There is a 14 minute gap between the two tracings. The effect of a 25 minute application of oxytocin is shown (minute 14 to minute 39 on the tracings); the response to oxytocin began to decline within 5-6 minutes. The slice was then washed with control solution for 10 minutes (minute 39 to minute 49), but the cell remained relatively unresponsive to both oxytocin and AVP. This cell had previously responded consistently to repeated applications of oxytocin and AVP.
of the Schaffer collateral pathway resulted in inhibition of the peptide induced activity of these cells (Figure 19C), indicating that they were pyramidal cells rather than interneurones (Lee et. al., 1980). No peptide sensitive cells were found in area CA3 or area CA4 or in the dentate gyrus, but these three regions were not explored extensively. There was no obvious difference in responsiveness between the left and right hippocampi or between the ventral and dorsal portions of the hippocampus.

Slice preparations from almost half of the rats studied contained no peptide sensitive cells, even though the slices often appeared to be as healthy as the more responsive slices when judged by the usual electrophysiological criteria (Dingledine et. al., 1980). The cause of this variation was not determined - the difficulties in determining why some slices fail to "work" have been discussed by Schwartzkroin (1981) - but it seems likely that the lack of responsiveness was a condition of the slices themselves, perhaps the result of trauma during cutting, rather than a property of the rat from which the slices were taken. In particular, the peptide sensitivity did not depend on prior exposure of the rat to AVP, since slices prepared from HoDI rats (2 female rats, 180-190 g) were as responsive as those from Wistar rats (41 male rats, 160-360 g). Furthermore, one Wistar rat was injected with AVP (1 µg, icv) two days prior to the experiment and should therefore have been sensitized to the peptide (Kasting et. al., 1980), but no responsive cells were found in the slices from this rat. The response may be temperature dependent, since slices maintained at room temperature (8 rats), rather than at 33°C, were insensitive to the peptides. No peptide sensitive cells were found in slices maintained in 1.0 mM Ca⁺⁺ (1 rat) or in 1.0 mM Ca⁺⁺ with 6.25 mM K⁺
(1 rat), but this finding may be coincidental, since the slices in these two preparations appeared to be in relatively poor condition. Slices tested in 1.5 mM Ca\(^{++}\) (1 rat) did not appear less responsive than those in 2.0 mM Ca\(^{++}\) (40 rats).

2. **DISCUSSION**

**Comparison of Iontophoretic and Superfusion Studies**

The excitation of rat hippocampal neurones by oxytocin and vasopressin applied *in vitro* demonstrates that these peptides can act directly on brain tissue. The results confirm, moreover, that the excitatory responses observed during iontophoresis of vasopressin were due to the peptide and not to the ejection of H\(^{+}\) ions or to nonspecific current effects. There were minor differences however, between the effects observed with iontophoresis and those observed with superfusion: (1) The response was more pronounced when the vasopressin was applied by superfusion. This was not an unexpected finding, since the amount of vasopressin released from the micropipettes was relatively small, and since the peptide would be largely confined to the area of the cell body. Superfusion, on the other hand, would allow the peptide to reach the dendrites, where the fibers immunoreactive for vasopressin are reported to synapse (Buijs et. al., 1978; Buijs and Swaab, 1979). (2) There was a difference in the time course of the response: the fact that the activity of the cells often began to increase within seconds when vasopressin was applied by iontophoresis (Figure 8) indicates that the delay observed with superfusion was due almost entirely to the time required for the peptide to reach the cell. (3) Repeated iontophoresis of vasopressin often resulted in a relatively
rapid decline in the magnitude of the response (Figure 11), an effect not observed when the peptide was applied by superfusion. This difference was a characteristic of the method of applying the peptide rather than the type of preparation, since iontophoresis resulted in a decline both in vivo and in vitro, and it therefore seems likely that the decline was due to progressive blockage of the micropipette tip rather than to tachyphylaxis. Alternatively, the local concentration of vasopressin at the cell body may be higher during iontophoresis than during superfusion, resulting in a more rapid decline in responsiveness.

Nature of the Hippocampal Receptors

Confirmation that the response to the NHP peptides was mediated by specific receptors was found in the ability of two vasopressin antagonists to block the response. The antagonist d(CH$_2$)$_5$Tyr(Me)AVP (Kruszynski et. al., 1980) blocks the response to vasopressin at the VP (vasopressor) receptor but has negligible activity at the AD (antidiuretic) receptor, while d(CH$_2$)$_5$-D-Tyr(Me)VAVP (Manning et. al., 1982) is a potent antagonist at both receptors (Table II). Since both antagonists were able to block the excitations in the hippocampal slice, the responses could not have been mediated by the AD receptor. Oxytocin and Gly$^7$OT, which are relatively inactive at the vasopressin receptors (Berde and Boissonnas, 1966; Lowbridge et. al., 1977; Cash, 1978), may have been acting on oxytocin receptors similar to those in the uterus or the mammary gland (the OT and the ME receptors, respectively). This would not be inconsistent with the ability of the vasopressin antagonist d(CH$_2$)$_5$Tyr(Me)AVP to block the hippocampal response to oxytocin, since this antagonist has also been reported to block the action of oxytocin on the rat uterus in vitro.
The effect of \( d(CH_2)_5-D-Tyr(Me)VAVP \) on OT and ME receptors has not been reported.

It seems more likely, however, that oxytocin and vasopressin were both acting on the same population of receptors. This interpretation is supported by the fact that the hippocampal neurones were never found to distinguish between the two peptides; cells responsive to either of these peptides were always found, when tested, to be about equally responsive to the other. The demonstration that continuous treatment with oxytocin rendered a cell refractory to both oxytocin and vasopressin also indicates a common receptor for the two peptides. If this is the case, the hippocampal receptor must be different from the endocrine NHP peptide receptors listed in Table II, all of which are relatively selective for oxytocin or vasopressin. There is a considerable body of evidence, summarized in Chapter I of this thesis, that additional types of NHP peptide receptor are present in mammalian tissues, but detailed information about these receptors is lacking. The specificity of the hippocampal receptor is similar to that of the NHP peptide receptor in the nervous system of the snail, in that both receptors respond to oxytocin, AVP, LVP, and AVT but not to PLG or DGLVP (Barker et. al., 1975).

There is also evidence that the NHP peptides are converted to smaller fragments by enzymes in the brain (Burbach et. al., 1980; Burbach and de Wied, 1981), and that these fragments are responsible for at least some of the effects of the peptides (Flexner et. al., 1978; Walter et. al., 1975; 1978). Burbach et. al. (1980) have reported that oxytocin can be cleaved by enzymes found in a synaptosomal plasma membrane preparation of rat limbic brain tissue. Both aminopeptidase activity and "C-terminal
cleaving peptidase" activity were found. The C-terminal peptidase, which was present in only small amounts, released glycinamide and Leu-GlyNH₂ from oxytocin. It seems unlikely, however, that the excitations observed in the present study were caused by active fragments. If the peptides were converted to active fragments by an aminopeptidase, for example, one would expect the enzyme resistant analogue ddOT to be less active than oxytocin; in fact, the opposite was found to be the case. Cleavage of the C-terminal portion of the peptides would be equally unlikely to produce active fragments, since removal of even a single C-terminal residue from AVP resulted in a fragment, DGAVP, almost totally lacking in activity. Oxytocin can be split at the 6-7 bond by an enzyme present in the rat median eminence (Celis et. al., 1971), but not yet detected in the hippocampus, to produce tocinoic acid (OT₁₋₆) and PLG (OT₇₋₉). Tocinoic acid was not tested in the present study, but PLG was found to be totally inactive. Both PLG (Kastin et. al., 1976) and DGAVP (Abood et. al., 1980) alter behavior in the intact rat, but they must bind to receptors different from those studied in the present experiment. Tritiated PLG injected into the lateral ventricle of the rat has been shown to bind selectively to hippocampal tissue (Pelletier et. al., 1975), but the label was largely confined to the cell body layer, while the terminals immunoreactive for oxytocin and vasopressin are found primarily in the dendritic region (Buijs et. al., 1978; Buijs and Swaab, 1979).

**Effect of Somatostatin**

The peptide somatostatin (SST), at a concentration of 0.05-0.5 µM, was also found to excite hippocampal neurones. The fact that SST excited a somewhat different population of neurones from the NHP peptides confirms
that SST must have been acting on a different set of receptors. Dodd and Kelly (1978) have reported that 3 mM SST applied by iontophoresis, by pressure injection, or in the form of droplets caused rapid depolarization and an increase in excitability of CA1 cells in the rat hippocampal slice. Pittman and Siggins (1981), on the other hand, found that 0.12-1.2 µM SST added to the bath solution inhibited spontaneous activity in the rat hippocampal slice; 9 of 10 pyramidal cells were hyperpolarized by the peptide. The difference in response cannot be attributed to the higher concentration used by Dodd and Kelly, since excitations were observed in the present study with concentrations similar to those used by Pittman and Siggins. Inhibitions would not have been detectable in the present study because of the lack of spontaneous activity.

Relation of Excitations to Biochemical Changes

There have been reports that oxytocin and vasopressin affect the release of serotonin, dopamine, and norepinephrine from nerve terminals in several areas of the rat brain, including the hippocampus and the septum (Ramaekers et. al., 1977; Schwarzberg et. al., 1981). This response is probably not involved in the excitations observed in the present experiment, however. The serotonergic and catecholaminergic inputs are severed in the slice preparation, and the NHP peptides are reported to be incapable of increasing the spontaneous efflux of these substances from inactive terminals in vitro. For example, in a study of rat hippocampal slices by Auerbach and Lipton (1982), AVP did not affect spontaneous serotonin release, even though the same concentration of AVP (10⁻³-10⁻⁶ M) increased the rate of K⁺ induced release. Furthermore, the effect of AVP on K⁺ in-
duced serotonin release was confined to the dentate area, the serotonergic projection to CA1 being unaffected. Similarly, Starr (1982) has reported that oxytocin and vasopressin attenuated the $K^+$ induced release but not the spontaneous release of $^3$H-dopamine from rat striatal slices.

The ability of AVP to increase the corticosterone binding capacity of hippocampal tissue (Veldius and de Kloet, 1982) would also seem to be unrelated to the excitation of hippocampal neurones, since the binding capacity was also increased by DGAVP, which was inactive in the present experiments, and was not increased by oxytocin.

**Effect of NHP Peptides on Hippocampal Activity**

Gähwiler (1978) has studied the effect of the NHP peptides on cultured neurones from several areas of the rat brain including the hippocampus and the hypothalamus. Oxytocin, applied in the bathing medium at a concentration of $10^{-5}$ M, was found to excite 3 of 9 hippocampal cells but did not affect cells from the supraoptic area or the hypothalamus. The excitation of hippocampal cells by oxytocin is in agreement with the present study, although the cultured cells appear to be less responsive than those in the hippocampal slice. LVP, at $10^{-7}$ to $10^{-6}$ M, caused a rapid, reversible inhibition of the hypothalamic cells but, in contrast to the present findings, did not alter the activity of cells from the hippocampus. Cells from the cerebellum and from the locus coeruleus were also unaffected by the peptide. The lack of effect of LVP on cultured hippocampal cells is difficult to evaluate, however, since LVP was not tested in the same concentration as oxytocin. SST was found to have no effect on the cultured hippocampal or hypothalamic cells at a concentration of $10^{-5}$ M, although
hippocampal cells were excited by $5 \times 10^{-7}$ M SST in the present study.

Since the present work was begun, the results of a similar study of rat hippocampal slices have been published by workers in Geneva (Mühelethaler et. al., 1982; Dreifuss and Mühelethaler, 1981, 1982a). Vasopressin was found to increase the spontaneous activity of CA1 neurones, and the response could be blocked by the antagonist $d(CH_2)_5$Tyr(Me)AVP. The concentrations of peptide ($10^{-8}-10^{-6}$ M) and the time course and magnitude of the responses were comparable to those reported in this thesis. Also in agreement with the present study, repeated applications of peptide resulted in little or no tachyphylaxis - whether tachyphylaxis occurred with continuous application was not reported; and HoDI rats were found to be as responsive as normal rats. The medium used by Mühelethaler et. al. (1982) had a low Ca$^{++}$ and Mg$^{++}$ content (1.0 mM Ca$^{++}$; 1.4 mM Mg$^{++}$) and a high K$^+$ content (6.24 mM) compared to the medium used in the present experiment (2.0 mM Ca$^{++}$, 2.0 mM Mg$^{++}$, and 5.0 mM K$^+$). As a result, the cells in their preparations had a greater spontaneous firing rate (mean 13 spikes per second), the activity occurring as intermittent bursts. Fifty-six (89%) of 63 spontaneously active cells were reported to respond to AVP and LVP.

Mühelethaler et. al. (1982) were primarily concerned with the effects of vasopressin rather than of oxytocin, and their investigation was therefore confined to the ventral hippocampus, the dorsal hippocampus being relatively devoid of vasopressin containing terminals (Buijs et. al., 1978). Oxytocin was also tested and was found to excite 5 of 6 cells, but the effect of the antagonist on oxytocin induced excitations was not tested and no oxytocin analogues were studied. The N-terminal vasopressin fragment DGAVP and the corresponding C-terminal fragment glycinamide were
reported to be inactive. The weak excitations produced by DGAVP in the present experiments would probably not have been detected in their study because of the high level of spontaneous activity. The specific AD receptor agonist DDAVP, which was not tested in the present experiment, was found by Mühlethaler to be much weaker than AVP or LVP. The two studies are therefore in agreement that the response was not mediated by the AD receptor, and the lack of response to DDAVP would seem to preclude the HF receptor as well (Table II).

Mühlethaler et. al. (1982) demonstrated that the excitations produced by the peptides were not due to a presynaptic action, since the response was not blocked by propanolol, atropine, or naloxone or by abolishing all synaptic activity with 10 mM Mg++. Intracellular studies (Dreifuss and Mühlethaler, 1981; Mühlethaler and Dreifuss, 1981) indicated that vasopressin caused a slight depolarization and a small increase in conductance in a few of the excited cells.

The fact that oxytocin and vasopressin were found to excite cells in the present study does not necessarily mean that the physiological function of these peptides is excitatory. As mentioned above in relation to SST, which excites hippocampal neurones in some laboratories and inhibits them in others, the nature of the response may depend on the experimental conditions. In the present experiment, inhibitions would not have been detected because of the low level of spontaneous activity in the NHP sensitive cells, but Mühlethaler and Dreifuss (1981) report that in their intracellular studies vasopressin was found to inhibit almost as many cells as it excited. Furthermore, they have reported in a more recent study (Dreifuss and Mühlethaler, 1982b) that the cells excited by oxytocin
and vasopressin appeared to be interneurons. Pyramidal cells were either inhibited or unaffected by the peptides, and intracellular recordings indicated that the inhibitions were the result of excitation of inhibitory interneurons. This finding is not incompatible with their earlier report that 89% of spontaneously active cells were excited by vasopressin, since the intermittent bursting activity of these cells suggests that they were probably interneurons (see Dreifuss and Mühlethaler, 1982b). It is not clear, however, why they found most cells to be excited in their earlier intracellular work (Dreifuss and Mühlethaler, 1981), since one would expect most of the impaled cells to be pyramidal cells, and it is difficult to reconcile their findings with the present work, in which the neurones excited by the peptides were clearly pyramidal cells (Figure 19). The most obvious difference in the techniques used in the two laboratories is in the composition of the medium, which contained 2.0 mM Ca$^{++}$ in the present study and in some of the intracellular work by Mühlethaler and Dreifuss (1981), but contained 0.8-1.0 mM Ca$^{++}$ in their extracellular studies (Mühlethaler and Dreifuss, 1981; Mühlethaler et. al., 1982). It is possible that the pyramidal cells are excited by vasopressin when the Ca$^{++}$ concentration is high, but that the interneurons are excited when the Ca$^{++}$ concentration is low. The low Ca$^{++}$ concentration may also be the cause of the pronounced spontaneous activity of interneurons reported by Dreifuss and Mühlethaler (1982b).

If the inhibition of the pyramidal cells in the low Ca$^{++}$ medium was caused by the excitation of interneurons, it is possible that the excitation of the pyramidal cells in the present work was secondary to inhibition of the interneurons. Alternatively, it is possible that the peptides
have a direct excitatory effect on both the pyramidal cells and the inter-
neurones, and that the net effect on pyramidal cell activity is a balance
between the direct excitation and the inhibition produced by increased
interneurone activity. Moreover, there have been reports that the inter-
neurones may have both excitatory and inhibitory effects on pyramidal
cells in vitro (Swanson et al., 1982, page 640).

CSF Peptides and Hippocampal Function

It is unlikely that endogenous oxytocin and vasopressin in the CSF
would cause excitation of neurones in the CA1 region of the hippocampus,
since the lowest concentration of peptide producing a measurable excita-
tion in the slice preparation ($10^{-9}$ M) was about 10-100 fold greater than
the concentration of the peptides in the rat CSF (Dogterom et al., 1977).
The CSF borne peptides could produce significant effects only if the
neurones are more responsive in vivo than in vitro or if the concentration
of the peptides in the CSF is drastically increased under some circumstances,
such as during febrile convulsions (Kasting et al., 1980). The fact that
the neurones appeared to be unable to distinguish between oxytocin and
vasopressin would also argue against a role for the CSF borne peptides.

Relation of Hippocampal Excitation to Behavioral Changes

There is considerable evidence that some of the behavioral changes
produced by injection of oxytocin or vasopressin may involve the hippo-
campus (Kovács et al., 1982; Ramaekers et al., 1977), and the peptide
sensitivity of hippocampal neurones demonstrated in the present in vitro
experiments would be consistent with such a suggestion. Intraventricular
injection of 1.0 ng of vasopressin, the dose most frequently used in
studies of conditioned behavior, would result in a concentration greater than $10^{-9}$ M in the CSF, and the 100 ng injections used to produce convulsions in rats (Abood et. al., 1980) would yield a concentration in excess of $10^{-7}$ M. Since these concentrations are comparable to those found to excite hippocampal neurones in vitro, it would not be unreasonable to suspect that the response to the centrally administered vasopressin might be the result of peptide induced excitation of hippocampal neurones.

An excitation of hippocampal neurones could, for example, explain the convulsions produced by icv injections of vasopressin. The major difficulty with this proposal is that oxytocin, which is as effective as vasopressin in exciting hippocampal neurones, has little or no convulsant activity (Abood et. al., 1980). Similarly, the ability of vasopressin to prolong retention of conditioned behavior cannot be explained by the excitation of hippocampal CA1 neurones, since oxytocin, which produces the same excitation, does not produce the same changes in conditioned behavior. It seems more likely, in fact, that the prolonged retention involves the dentate gyrus of the hippocampus rather than the CA1 region.

It has been reported that AVP injected into the dentate gyrus facilitates retention of avoidance behavior, while oxytocin has the opposite effect (Kovács et. al., 1979b), and that anti-AVP serum, injected into the dentate gyrus, impairs retention of avoidance behavior (Kovács et. al., 1982). It is somewhat disturbing however, that the photograph used to indicate the cannula placement in the latter study shows that the cannula was in the CA3 region rather than in the dentate gyrus. The antiserum released from the cannula, traced by an immunoperoxidase technique, reached CA3 neurones and possibly some CA1 and CA4 neurones and spread through the
ventricle to the lateral septum but did not reach the granule cell layer of the dentate. AVP promotes serotonin release from the dentate gyrus but not from the CA1 region (Auerbach and Lipton, 1982), and there is indirect evidence that the hippocampal serotonin turnover is involved in the memory related actions of vasopressin (Ramaekers et. al., 1977).

The enhancement of certain spontaneous behaviors (hyperactivity, grooming, and foraging) in mice is quite similar in its peptide specificity to the excitation of CA1 neurones (Delanoy et. al., 1979). Oxytocin, vasopressin, and vasotocin were found to be strong agonists in both systems, while DGAVP and DDAVP were weak or inactive. It is therefore possible that the changes in spontaneous behavior result, at least in part, from a change in neuronal activity in the CA1 region of the hippocampus. The significance of this behavioral response is unknown.

Conclusions

The present study demonstrates that oxytocin and vasopressin can cause rapid and profound changes in the activity of neurones in the CA1 region of the rat hippocampus. The nature of the response, excitation or inhibition, depends on the experimental conditions, and it is possible that the response of the pyramidal cells is secondary to changes in interneurone activity. Determining the relation of the in vitro response to the physiological actions of these peptides will require further study.

There is apparently more than one type of NHP peptide receptor in the hippocampus, since the receptor(s) responsible for the excitation of CA1 cells appear to be different from the receptor which mediates the
effect of vasopressin on serotonin release in the dentate gyrus. The PLG binding site present on CA1 neurones (Pelletier et al., 1975) may represent an additional type of NHP peptide receptor, the function of which is unknown. A comparison of the peptide specificity observed in the present experiment with the specificity reported in other studies suggests that the convulsive and memory-related actions of vasopressin do not directly involve the CA1 region and may be related instead to the control of serotonin metabolism in the dentate gyrus. The excitation of CA1 neurones by the NHP peptides may be involved in the reported effects of these peptides on spontaneous behavior in mice.
CHAPTER IV. OXYTOCIN, VASOPRESSIN AND CNS FUNCTION

It has not been proven that oxytocin and vasopressin are involved in synaptic transmission. The criteria by which a substance is established as a mediator of synaptic transmission are the subject of some debate, since some of the criteria originally described for studies of small molecules may not be entirely relevant to peptides (Werman, 1972; Burnstock, 1976; Barchias et. al., 1978), but useful evidence would certainly include the demonstration that (1) the substance is present in and released from synaptic terminals, and (2) that the effect of the endogenous substance can be imitated by exogenous application and blocked by agents that antagonize the exogenous application.

That oxytocin and vasopressin are present in nerve terminals in many areas of the CNS has been well established by immunocytochemical and radioimmunoassay studies, but release has been demonstrated only in the septum (Cooper et. al., 1979). Injection of AVP into the septum of sheep reduces the febrile response to endotoxin, while injection of a vasopressin antagonist has the opposite effect (Cooper et. al., 1979; Kasting et. al., 1979). This finding suggests that the release of endogenous AVP may have an antifebrile action in the septum. Iontophoresis of AVP was found to excite septal neurones in the present experiment, but it is not certain that the population of neurones excited by iontophoresis of AVP is involved in the antifebrile response, and iontophoresis of an antagonist was not attempted. The actions of oxytocin and vasopressin applied to the hippocampus have been described both in the present study and in the work of Mühlethaler et. al. (1982), but while the peptides are known to be present in nerve terminals in the hippocampus (Buijs, 1980), the circumstances under which they are released and the response
to endogenous release are entirely unknown.

Pittman et. al. (1981) have shown that cells in the PVN can be antidromically activated by stimulation of the areas known to contain immunoreactive terminals, such as the lateral septum, the amygdala, and the periaqueductal gray. If stimulation of the antidromically identified cells could be shown to produce a measurable response in the target area, it might be possible to demonstrate that the response could be imitated by exogenous peptide applied, for example, by pressure injection, or that it could be blocked by local application of an antagonist. High frequency stimulation might be necessary to release enough peptide to produce a significant response. It is not obvious, however, what type of response should be measured; one might be able to detect a change in unit activity, a modification of the response evoked by stimulation of a third area, or a biochemical or trophic change.

That the changes produced by the peptides may be more subtle than simply excitation or inhibition is indicated in a report by Chepkova (1981), who studied the effect of LVP on long term potentiation (LTP) of the Schaffer collateral pathway in mouse hippocampal slices. The LVP was added to the bath at a concentration of 1-5 μM for a period of 30-60 minutes, and LTP was induced by tetanic stimulation applied during this period. The peptide did not increase the initial magnitude of the LTP, measured as the change in amplitude of the CA1 population spike, and in most cases did not affect the size of the population spike measured prior to the LTP. The peptide did appear, however, to prolong the duration of LTP; that is, in slices treated with the peptide, the amplitude of the potentiated population spike declined more slowly than in untreated
slices. Chepkova noted the similarity between this response and the reported ability of vasopressin to prolong the duration of conditioned behavior. Unfortunately, the experiment did not establish that the action of the peptide was actually related to LTP, since the population spike normally declines in vitro even in unpotentiated slices. To establish that vasopressin prolongs LTP, it would be necessary to show that it does not slow the decline of a second, unpotentiated pathway. It would also be helpful to demonstrate that the effect of the vasopressin on LTP could be blocked by a vasopressin antagonist.

Whether the actions of oxytocin and vasopressin in the CNS are related to their endocrine actions is not known. If the fibers projecting to the various portions of the CNS are from the same cells that project to the posterior pituitary - such a possibility is suggested by the bipolar and multipolar shape of the cells (Defendini and Zimmerman, 1978) - then the peptides will be released into these areas under the same circumstances under which they are released into the blood. If that is the case, the CNS functions must be related to the endocrine functions. Buijs (1980) has pointed out that many of the areas innervated by fibers containing vasopressin, such as the OVLT, the periventricular nucleus, and the choroid, are thought to be involved in water balance. Terminals immunoreactive for vasopressin have also been found in areas concerned with cardiovascular function (Sofroniew and Schrell, 1981). Similarly, the promotion of maternal behavior by oxytocin (Pedersen et al., 1982) would seem to be a reasonable action for a hormone released during suckling. On the other hand, if the CNS projections are from a population of neurones distinct from those projecting to the posterior pituitary, then
It is possible that the release of peptide into synapses in the CNS is unrelated to the endocrine action of the peptides. While this point is still controversial, electrophysiological experiments by Pittman et. al. (1981) indicate that the PVN cells projecting to the lateral septum, the amygdala, and the periaqueductal gray do not project to the pituitary. Similarly, histological studies suggest that the PVN cells projecting to the medulla and the spinal cord are separate from those projecting to the pituitary (Swanson and Sawchenko, 1980).

It was suggested in the introduction to this thesis that an understanding of the functions of vasopressin and oxytocin in the CNS would be facilitated by a more detailed knowledge of the actions of the peptides in individual target areas. In particular, differences in the responses to injections of AVP, oxytocin, DGLVP, and PLG suggest that there may be regional differences in peptide specificity. The in vitro slice preparation was found in the present study to be a satisfactory system for investigating the effects of the peptides in the hippocampus and would probably be equally useful in studying other areas, such as the septum, the amygdala, the medulla, the spinal cord, and the hypothalamus. The response can be localized much more accurately in vitro than is possible with microinjection techniques. In addition, synthetic analogues can be screened for CNS activity much more rapidly in vitro than in vivo.
REFERENCES


